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THE FORMATION OF d-GLUCONIC ACID BY *BACTERIUM SAVASTANOI* SMITH.

By CARL L. ALSBERG.

(*From the Office of Drug Plant, Poisonous Plant, Physiological and Fermentation Investigations, Bureau of Plant Industry, Department of Agriculture.*)

(Received for publication, December 8, 1910.)

Dr. Erwin F. Smith found that the olive tubercle organism, *Bacterium Savastanoi*, studied and named by him, forms an acid when grown aerobically in the presence of certain sugars. Some time ago Dr. Smith requested me to attempt the identification of the acid formed in the presence of glucose.[1] To that end he supplied me with four flasks of these organisms grown during five months at room temperature upon a medium consisting of 14 grams of Witte peptone, 28 grams of calcium carbonate, 20 grams of Merck's dextrose and 1000 cc. of filtered tap water. It is therefore to Dr. Smith's courtesy that I am indebted for the opportunity to make the study herein recorded.[2]

The calcium carbonate, found to be free from insoluble organic calcium salt, was removed from the culture by filtration. The clear filtrate was concentrated to a thin syrup. It was set aside and the walls of the vessel occasionally rubbed with a glass rod. In this way an abundant, white, cauliflower-like mass developed, that under a hand lens could be seen to consist of aggregates of fine needles. The crystals were drained under pressure from the mother liquor before recrystallizing from hot water. The mother liquor from the flasks first used reduced Fehling's solution powerfully, the crystals did not. The flask used last, which held a culture two months older, contained no reducing material. By repeated recrystallizations white crystals were easily obtained without the use of bone-black. They showed great tendency to form supersaturated solutions, so that it was often necessary to inoculate with a few crystals to obtain a satisfactory crystallization. The purified preparation was then dried under diminished pressure over sulphuric acid at room temperature. When at constant weight it was further dried in an air bath at a temperature rising gradually to 115° C., when it lost nothing more, so that if water of crystallization were present, as claimed by some, it must have been removed at room temperature under diminished pressure over sulphuric acid. The calcium content was then determined by incinerating and heating to constant weight. It proved to be 9.36 per cent. This corresponds to anhydrous calcium d-gluconate which contains theoretically 9.35 per cent calcium. Thereupon the acid was set free by precipitating the calcium with oxalic acid, and removing the calcium oxalate. The solution was extracted with ether to remove any slight excess of oxalic acid, and evaporated to a syrup. There was some indication of crystallization in the syrup, although crystals were not abundant. d-Gluconic acid does not crystallize, but the crystallizing lactone forms easily.[1] From the free acid the cinchonine salt was prepared. This had the characteristic appearance and solubilities of cinchonine d-gluconate.[2] When the acid was treated with a faintly acid solution of ferric chloride a fine yellow color was obtained, a reaction which d-gluconic acid shares with a number of other oxy-acids.[3] From the calcium salt the hydrazide was prepared as recommended by Fischer.[4] Here, too, the tendency to form supersaturated solutions delays crystallization. After three recrystallizations from hot water the hydrazide showed the characteristic behavior and melting point of d-gluconic acid hydrazide.[5] The hydrazide dissolved in strong sulphuric acid gave a red coloration with ferric chloride, as shown by Bülow's Reaction.[6]

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[1] Erwin F. Smith: Recent Studies of the Olive Tubercle Organism. Bull. No. 131, Part iv, Bureau of Plant Industry, U. S. Dept. of Agriculture, Washington, D. C. 1908.

[2] Cf. *Proc. Amer. Chem. Soc.*, Feb. 11, 1909, p. 59; *Proc. Soc. of Exp. Biol. and Med.*, 1909, ii, p. 83.

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[1] E. Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxiii, p. 2625.

[2] Lippmann: *Chemie der Zuckerarten*, p. 318.

[3] Berg: *Bull. de la soc. chim.*, [3] xi, p. 882.

[4] Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxiii, p. 799.

[5] Fischer: *Ibid.*, xxiii, p. 799.

[6] Carl Bülow: Ueber einige Verbindungen des Phenylhydrazines, *Ann. d. Chem. u. Pharmacie*, ccxxxvi, p. 194.

The optical activity was determined as recommended by Fischer.[1] Twenty-five centigrams of the calcium salt gave a rotation of +1.50° in a 100 mm. tube. Fischer obtained +1.55° under these conditions.

There can therefore be no doubt that the acid obtained from the cultures was d-gluconic acid. This acid has been obtained before as the result of the action of other organisms upon glucose;[2] but this, so far as I am aware, is the first record of its production by a pathogenic organism.

At the beginning of this investigation it was assumed that the reducing substance remaining in the mother liquors after the crystallization of the calcium d-gluconate was glucose. Since Boutroux,[3] however, has described an organism which is capable of making an oxy-gluconic acid with reducing power from either glucose or d-gluconic acid, it is possible that the reducing material in the cultures of *Bacterium Savastanoi* Smith is a further oxidation product of d-gluconic acid. Some of the filtered culture fluid, consequently, was treated with 95 per cent alcohol until further additon of alcohol caused no more precipitation. The crumby white precipitate formed was filtered off, redissolved in a small volume of water, and reprecipitated with alcohol. The precipitate then no longer reduced Fehling's solution. All the reducing material had passed into the alcoholic filtrates. The alcohol was removed under diminished pressure at a temperature below 50°. A sample of the solution after having been freed from alcohol in this way was readily fermented by yeast so that its reducing power quite disappeared. It still contained a little calcium d-gluconate. From the unfermented solution the osazone was obtained in the usual way. The osazone was separated from the hydrazide of d-gluconic acid by means of its greater insolubility in hot water. It was recrystallized until its melting point remained contant at 204°. There can therefore be no doubt that the reducing substance was unfermented glucose. This was subsequently confirmed by the fact, already mentioned, that older cultures lost their reducing power.

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[1] *Ber. d. deutsch. chem. Gesellsch,* xxiii, p. 2611.

[2] The literature has been collected by Lippmann: *Chemie der Zuckerarten,* pp. 323 and 431.

[3] Boutroux: Sur une fermentation acide du glucose, *Compt. rend. de l'Acad. des sci.,* cii, p. 924; cxi, p. 185.

The loss of reducing power did not seem to be accompanied by any considerable loss of d-gluconic acid as far as can be judged from the large yields of calcium d-gluconate obtained from these old cultures.

From a seven-months-old culture which no longer contained any of the 20 grams of glucose originally put into it, 15.93 grams of calcium d-gluconate were obtained. As this salt could not be separated quantitatively from the mother liquor, the amount remaining in solution was estimated by determining the calcium in the mother liquor. Before incineration the solution was boiled and filtered to remove any calcium bicarbonate that may have been in solution. The calcium in solution corresponded to 7.33 grams of calcium gluconate, so that 21.32 grams of d-gluconic acid had been formed corresponding to 17.02 grams of glucose. The deficit was less than 3 grams of glucose. The glucose used was Merck's anhydrous glucose. In preparing the culture medium it was taken directly from the bottle without further drying. It may not have been quite anhydrous and, if so, the amount of glucose unaccounted for may be even less. At any rate it is justifiable to conclude that only a little glucose was converted into anything other than d-gluconic acid. There may be a small error in this calculation due to the fact that the assumption is made that the calcium remaining in the mother liquor is combined with d-gluconic acid and that other soluble calcium salts are not present. Certainly no other acid was found.

The amount of energy liberated by the oxidation of glucose to d-gluconic acid is very considerable. The heat of combustion of glucose is 673.7 Cal. I have not been able to find data on the heat of combustion of d-gluconic acid; but that of the lactone of l-gluconic acid is 615.3 Cal.[1] The heat of combustion of d-gluconic acid will be different, though probably not very much. For want of better data, I have assumed that it is the same. Then the heat of reaction in the conversion of glucose into d-gluconic acid is probably about 58 Cal. or 8.6+ per cent of the total energy obtainable by the complete combustion of glucose. This is of the same order of magnitude as some other fermentations such as the formation of alcohol.[2] Inasmuch as about 17.02 grams of glucose have been oxidized, it follows that about 5.48 Cal. have been liberated by the organisms in a single flask in the course of seven months or an average of .026 Cal. per day, provided we assume that it is permissible to consider only the simplest fermentation equation. That such calculations are very inaccurate either because they disregard the heats of solution or because the fermentation equations are incomplete as well as for a variety of other reasons has been shown by Rubner.[1] Still for the determination merely of the order of magnitude of the energy converted in the formation of d-gluconic acid it is accurate enough, particularly as it represents minimum not maximum values. It is possible that there is still another source of error in this calculation due to the fact that all the glucose may have been converted some days or even weeks before these determinations were made. However, as this error if present would make the per diem values smaller, not larger, it can not impair the argument. Moreover, this calculation does not take into consideration the 3 grams of glucose unaccounted for, which were probably also oxidized to furnish heat as they could not be stored as reserve carbohydrate in the small amount of micro-organisms present. Even an accurate knowledge of the energy converted would be of very little value without a knowlede of the mass of living matter concerned in the process. To weigh the micro-organisms in liquid culture media accurately is as yet imposible.[2] Results, sufficiently accurate for the approximations necessary in this discussion, were obtained thus:—The organisms were decanted from the lime salts present in the medium. The remaining lime salts were suspended in much water and dissolved slowly with acid, without using an excess. The solutions were then filtered through asbestos in perforated crucibles. The residues were washed, dried, and weighed. The filtrates were centrifugated at high speed and the weight of the washed, dried sediment added to that of the Gooch crucibles. The dry weight of the micro-organisms was thus found to be 0.0087 gram, which can not be regarded as more than a very rough approximation. If we assume the organism to contain not over 85 per cent water[1] the live weight would be .058 gram. We must, however, further take into consideration that probably all organisms were not active at any one time. The organism is aerobic and perhaps only the surface pellicle was actively oxidizing. Those organisms which had dropped beneath the surface were probably not active, though they may have been alive. If such organisms were dead they might have been autolyzed setting enzymes free in the medium to continue oxidation outside the cells. This possibility deserves consideration in view of recent studies on bacterial oxidizing enzymes surviving the bacteria themselves.[2] No oxidation was found in a preliminary experiment with filtered culture containing an antiseptic, toluol. Possibly experiments of longer duration might reveal it. Moreover, in view of the great difficulty with which such intracellular enzymes are separated from the cells, and further, in view of their great instability when separated, any considerable oxidation by this means seems improbable. At any rate that it may occur remains to be proved. For non-oxidizing enzymes, a post-mortem action on the medium has been demonstrated in the ripening of cheese.[3]

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[1] Lippmann: *op. cit.*, p. 1742.

[2] M. Rubner: Calorimetrische Untersuchungen, *Zeitschr. f. Biol.*, xxi, p. 338.

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[1] M. Rubner: Die Umsetzungswärme bei der Alkoholgährung, *Arch. f. Hygiene*, xlix, p. 355 ff.

[2] M. Rubner: Energieverbrauch im Leben der Mikroorganismen *Arch. f. Hygiene*, xlviii, p. 288.

The amount of material metabolized and the amount of chemical energy converted is therefore very large when compared with the corresponding processes of higher forms such as germinating seeds or man. The *Bacterium Savastanoi* Smith under the conditions of these experiments according to this approximate calculation converts daily an amount of energy equivalent to at least 448.4 Cal. per kilo of organisms. Men require about 35 Cal. per kilo per day. The same fact has been brought out by Stoklasa. He found that 100 grams of dry *Clostridium butyricum* yielded 2.13 grams of carbon dioxide per hour; *Bacterium Hartlebii* 2.89 grams, sugar beet root 0.006 grams.[4] Rubner has also repeatedly drawn attention to this phenomenon and published fundamental investigations upon the metabolism of energy of micro-organisms.[1] The significance of these great quantitative differences between the metabolism of micro-organisms and that of larger forms of life are not yet quite clear. An attempt to offer an explanation for these differences and to point out their relations to theories of fermentation was made at the time this paper was presented. Its publication is reserved for another communication.

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[1] M. Rubner: Das städtische Sielwasser und seine Beziehung zur Flussverunreinigung, *Arch. f. Hygiene,* xlvi, p. 41.

[2] R. O. Herzog und A. Meier: Ueber Oxydation durch Schimmelpilze, *Zeitschr. f. physiol. Chem.*, lvii, p. 35; lix, p. 57.

[3] O. Jensen: *Landwirtschaftliches Jahrbuch der Schweiz,* 1906, p. 303.

[4] J. Stoklasa, J. Jelinck und E. Vitek: *Beitr. z. chem. Path. u. Physiol.* iii, p. 507.

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[1] M. Rubner: *op. cit;* M. Rubner: Grundlagen einer Theorie des Wachstums der Zelle nach Ernährungsversuchen an Hefe, *Sitzungsber. d. königl. preuss. Akad. d. Wissensch.*, 1909, p. 167; M. Rubner: Energieumsats im Leben der Spaltpilse, *Arch. f. Hygiene*, lvii, p. 193.

CHOLESTEROL BODIES IN SOILS: PHYTOSTEROL.[1]

By OSWALD SCHREINER and EDMUND C. SHOREY.

(Contribution from the Laboratory of Fertility Investigations, Bureau of Soils.)

(Received for publication, December 7, 1910.)

The increased interest of biological chemists in the lipoids has tended to direct greater attention to the cholesterol bodies that generally accompany them. This has resulted not only in considerable speculation regarding the physiological rôle of these bodies, but in important advances in our knowledge of the part these bodies play in the animal economy[2] and a greater understanding of the chemical constitution of these compounds.[3]

In plants a large number of cholesterol bodies occur.[4] These differ chiefly in melting point and crystalline form and are generally present in plant tissues in combination with higher fatty acids as esters.

In a previous paper[5] the isolation of a cholesterol body, agrosterol, from soils was described. This compound differed in melting point from any cholesterol previously described and the manner in which it was obtained indicated that it occurred in the soil as such and not in combination.

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[1] Published by permission of the Secretary of Agriculture.

[2] Doree Ellis and Gardner: *Proc. Roy. Soc.*, lxxx, 1908; lxxxi, 1909; Kusomoto: *Biochem. Zeitschr.*, xiv, pp. 411-416, 1908; Willstatter: *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 238, 1909.

[3] Diels and Abderhalden: *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 3177, 1903; Diels: *ibid.*, xli, p. 2597, 1908; Mauthner: *Monatsh. f. Chem.*, xxx, p. 635, 1909; Windaus: *Ber. d. deutsch. chem. Gesellsch.*, xli, pp. 611, 2588, 1908.

[4] Windaus and Hauth: *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 4378, 1906; xl, p. 3661, 1907; Jager: *Chem. Centralbl.*, 1907, i, pp. 13, 703; ii, p. 684; Bull. No. 53, Bureau of Soils, U. S. Dept. of Agric.; *Journ. Amer. Chem. Soc.*, xxxi, p. 116, 1909.

[5] *Journ. Amer. Chem. Soc.*, xxxi, p. 116, 1909; Bull. No. 53, Bureau of Soils, U. S. Dept. of Agric.

In the present paper the isolation of another cholesterol body from soils is presented. This has been identified as phytosterol, the most common cholesterol body of plants.

The phytosterol was obtained from a peaty soil containing 27 per cent of organic carbon. The soil was treated with boiling 95 per cent alcohol, the extract filtered while hot and allowed to cool. On cooling, a voluminous precipitate separated and was filtered off. This precipitate contained lignoceric acid described elsewhere.[1] The filtrate was carefully evaporated and kept at constant volume by the addition of water until the alcohol was removed. The reddish brown precipitate thus formed was separated by filtration, washed with water and dried. As thus obtained, it formed a brown, resinous powder. This material was extracted with boiling petroleum ether and on removing the ether from the extract, there was left a light-colored, oily mass which became semisolid on standing. This oily residue was saponified with alcoholic potash, the alcohol removed, the soap dried and extracted with boiling petroleum ether. The petroleum ether was removed from this extract, then treated with a relatively large volume of hot alcohol in which it was completely soluble. On cooling this solution, there was a separation of some microcrystalline material[2] which was removed by filtration and the alcohol allowed to evaporate. The residue thus obtained was dissolved in a small quantity of chloroform and allowed to stand. After a few hours crystals, having the appearance of phytosterol, were formed. The appearance of these crystals is shown in the photomicrograph and is characteristic of this body and quite distinct from cholesterol or agrosterol, the other cholesterol body found in soils.

The compound obtained in this way was purified by several recrystallizations. It melted at 135°, the melting point of phytosterol being given by different authorities from 132° to 138°. It gave the Liebermann cholesterol reaction strongly, as well as the cholesterol reaction with sulphuric acid and chloroform. The method by which this compound was obtained, its crystalline form, its melting point and color reactions, are sufficient to establish its identity as phytosterol.

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[1] *Journ. Amer. Chem. Soc.*, xxxii, p. 1674, 1910.

[2] Identified as hentriacontane, $C_{31}H_{64}$, *Journ. Amer. Chem. Soc.*, xxxiii, p. 81, 1911.

It will be noted that in this case the phytosterol was obtained as the result of saponification and it has not been possible to obtain this body from the soil under investigation by any method which did not involve saponification. The phytosterol then is no doubt present in the soil in combination, probably as an ester of a higher fatty acid, as in vegetable fats and oils. The well-known wide distribution of phytosterol in plants and the fact that when found in soil it is in combination, as in plants, make it probable that the phytosterol is a plant residue which remained unchanged in this soil.

While our knowledge of the physiological rôle of cholesterol in the animal body is as yet meager, still less is known of the part that such bodies play in the life of plants. The effect of the presence of cholesterol bodies in a soil or soil solution on the growth of plants or on the micro-flora of the soil is as yet a matter to be determined.

Both phytosterol and the agrosterol previously described occur in the soils in which they have been found, in very small quantities, but their presence establishes a connection with other theories of geological rather than biological interest. Several authorities have contended that the optical activity of certain mineral oils was due to the presence of cholesterol bodies.[1] While this has been disputed there has been much speculation regarding the source of the cholesterol bodies in mineral oils.[2]

The variability of the organic matter of soils has been pointed out and emphasized in previous papers, but the occurrence of these two cholesterol bodies, agrosterol and phytosterol, in soils shows that the variability extends to the compounds in one class. Phytosterol occurs in the soil in combination, probably as an unchanged plant residue, while agrosterol occurs free and may be the result of the breaking up of a similar combination or it may have been formed in the soil from fatty or other bodies of quite different constitution.

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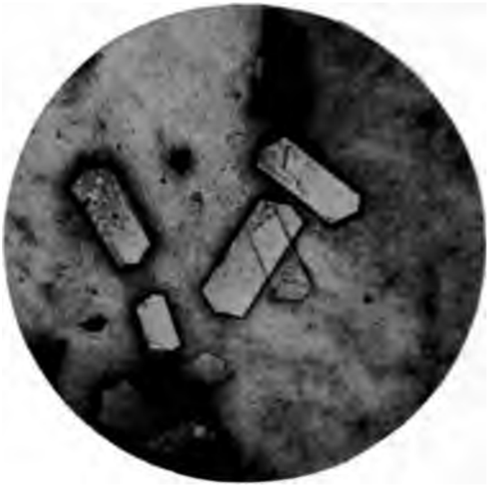
[1] Engler: *Chem. Zeit.*, 1906, p. 711; Rakusin: *ibid.*, p. 85; Marcussohn: *ibid.*, p. 419.

[2] Neuberg: *Biochem. Zeitschr.*, i, p. 368, 1906; Walden: *Chem. Zeit.*, 1906. p. 391.



PHYTOSTEROL OBTAINED FROM SOIL

THE INFLUENCE OF URETHANE IN THE PRODUCTION OF GLYCOSURIA IN RABBITS AFTER THE INTRAVENOUS INJECTION OF ADRENALIN.

By FRANK P. UNDERHILL.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Connecticut.)

(Received for publication, December 8, 1910.)

The vast accumulation of literature relative to adrenalin glycosuria contains very few records of attempts to demonstrate a quantitative relationship between adrenalin administration and sugar elimination. For the most part investigators have been content with the knowledge that a certain quantity of adrenalin injected subcutaneously or intraperitoneally almost invariably causes the appearance in the urine of significant quantities of dextrose. Moreover, it has been generally accepted that adrenalin given by mouth entirely fails to provoke glycosuria and that the intraperitoneal administration gives rise to a greater sugar excretion than the introduction of the drug directly into the circulation. What relationship exists between the quantity of adrenalin injected and the sugar eliminated, and how this relation may vary with change in the manner of adrenalin introduction are questions of some importance since adrenalin effects are constantly employed in the study of problems having a far reaching significance.

Perhaps the most suggestive investigation in this particular direction is the recent communication of Ritzmann[1]. He has shown that the degree of glycosuria is dependent upon the quantity of adrenalin present in the blood at any given moment. So long as adrenalin is present in the blood sugar in the urine is in order but glycosuria ceases as soon as adrenalin disappears from the circulation and almost immediately reappears when the drug is again introduced. Extremely dilute adrenalin solutions are potent in eliciting a relatively large excretion of sugar. There exists, according to Ritzmann, a direct relationship between the quantity of adrenalin introduced into the circulation and the amount of sugar eliminated in the urine, and for each rate of adrenalin injection there is a corresponding grade of glycosuria. Furthermore, adrenalin administered subcutaneously is not capable of inducing as much sugar to appear in the urine as a much smaller quantity of the drug introduced intravenously. In Ritzmann's experiments cats and rabbits were employed and the adrenalin was introduced into the jugular or femoral veins by a modification of the Kretchmer method. The injections were made with the animals under anaesthesia. In general when rabbits were used narcosis was produced with urethane given by stomach sound. Urine was collected through a permanent catheter.

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[1] Ritzmann: *Arch. f. exp. Path. u. Pharmakol.*, lxi, p. 231, 1909. See also Straub: *Münch. med. Wochenschr.*, 1909, No. 10; Pollak: *Arch. f. exp. Path. u. Pharmakol.*, lxi, p. 376, 1909.

In the course of an investigation it became desirable to make use of Ritzmann's observations and trials were made in order to determine whether in our hands entirely corroboratory results could be obtained. Our method of introducing the drug directly into the circulation of the experimental animal (the rabbit) was that indicated in a former paper,[1] that is, adrenalin (Parke, Davis and Company), suitably diluted with 0.9 per cent sodium chloride solution, was injected into the ear vein under pressure. This obviated the necessity of narcosis. Urine was obtained at the desired intervals by compression of the bladder through the body wall. Our experimental conditions conformed in every other respect with those of Ritzmann.

In spite of the harmony existing between the experimental conditions of Ritzmann's investigation and our own all attempts to provoke glycosuria by intravenous injections of dilute solutions of adrenalin into normal rabbits failed. Two experiments are given below in detail which will serve as typical examples of a large number of similar observations. Table 1 shows the results obtained in an attempt to duplicate Experiment 4 (p. 239) of Ritzmann while the data in Table 2 are those yielded in an endeavor to duplicate Ritzmann's Experiment 15 (p. 240). In Ritzmann's Experiment 4, 0.18 gram dextrose was present in the urine after the introduction of 75 cc. of 1:500000 adrenalin solution in 40 minutes, whereas in the present investigation, Table 1, no sugar appeared after nearly 200 cc. had been injected at the same rate. It is shown in Experiment 15 of Ritzmann's work that 0.28 gram sugar was present in the urine when 49 cc. of 1:250000 adrenalin solution had been injected in 20 minutes. Our introduction of 333 cc. (Table 2) of the same strength and at the same rate of injection did not induce any glycosuria.

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[1] Underhill and Closson: *Amer. Journ. of Physiol.*, xv, p. 321, 1906.

TABLE 1.

Female rabbit of 2400 grams weight. The urine in the bladder contained no reducing substances. Adrenalin solution, 1:500000.

TIME.	ADRENALIN INJECTED.	URINE EXCRETED.	REDUCTION TEST.*
	cc.	*cc.*	
11.22	0	0	
11.42	41	25	negative
12.02	35	10	negative
12.22	40	8	negative
12.42	32	10	negative
1.02	50	20	negative

*With Benedict's reagent. Benedict: *Journal of Biological Chemistry;* 1908, v. p. 485.

TABLE 2.

Male rabbit of 2200 grams weight. Urine in bladder did not reduce. Adrenalin solution employed, 1:250000.

TIME.	ADRENALIN INJECTED.	URINE EXCRETED.	REDUCTION TEST.
	cc.	*cc.*	
3.19	0	—	
3.39	49	0	negative
4.05	34	5	negative
4.27	35	20	negative
4.47	50	17	negative
4.58	15	7	negative
5.18	50	10	negative
5.39	50	18	negative
6.00	50	11	negative

In an endeavor to account for the discrepancy between Ritzmann's results and our own control experiments were carried through without suggesting a probable explanation. Finally observations were made upon animals that had received urethane by mouth. *All experiments in which sufficient urethane had been given yielded results in entire accord with those of Ritzmann.* Positive results were obtained invariably only when *sufficient* urethane had been introduced. The quantity of urethane necessary appears to be about one gram per kilo of body weight. Smaller quantities will, however, frequently furnish positive results but the smaller quantities cannot be relied upon, whereas with doses of one gram per kilo not a single experiment failed to induce glycosuria.

Table 3 presents data obtained upon an animal without urethane narcosis and in Table 4 may be found results from the same animal under urethane narcosis. Urethane alone is incapable of inducing glycosuria and negative results are also yielded when sodium chloride is introduced into a urethane narcotized rabbit in the quantities and at the rate employed in the adrenalin experiments. From these observations it would therefore appear that urethane renders the rabbit organism unusually sensitive to the glycosuria-inducing action of adrenalin. It has been demonstrated by Fröhlich and Loewi[1] that cocaine causes the organism of the cat and dog to become more sensitive to adrenalin with respect to its influence upon blood pressure, salivary secretion and mydriasis. It is possible

TABLE 3.

Male rabbit of 2000 grams weight. Bladder empty when tested. 1.5 grams urethane in 20 cc. water by stomach tube. Adrenalin solution employed, 1:250000.

TIME.	ADRENALIN INJECTED.	URINE EXCRETED	REDUCTION TEST.
	cc.	cc.	
3.25	0	—	
3.45	38	0	negative
4.05	33	0	negative
4.25	43	2	negative
4.45	42	3	negative
5.05	45	6	negative
5.25	45	26	negative
5.45	45	23	negative

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[1] Fröhlich and Loewi: *Arch. f. exp. Path. u. Pharmakol.*, lxii, p. 159, 1910.

that adrenalin plays a rôle in adrenalin glycosuria somewhat analogous to that of cocaine observed by the above mentioned authors. Moreover it is possible that other narcotics and anaesthetics may exercise an influence similar to that of urethane in this and other forms of experimental glycosuria.

In another portion of Ritzmann's paper there appears a comparison of the influence of adrenalin when administered subcutaneously and intravenously. It is shown that a very small quantity of adrenalin injected intravenously will cause very much more sugar to appear in the urine than a much larger quantity of adrenalin introduced subcutaneously. This comparison seems hardly fair in view of the influence of urethane noted above since in Ritzmann's experiments the narcotic was employed only when adrenalin was given intravenously. From a consideration of the above observations concerning the rôle played by urethane in the production of glycosuria after intravenous injections of dilute solutions of adrenalin it appeared desirable to determine whether in the non-narcotized animal a definite quantity of adrenalin injected intravenously in dilute solutions would yield more sugar in the urine than the same quantity of adrenalin administered subcutaneously in the dilution, 1:1000. Table 5 shows the results of four such experiments. The rabbits were maintained under constant conditions of diet throughout so that the divergences in sugar elimination can not be ascribed to such an origin. Neither can they be attributed to lack of glycogen in the body since a lapse of time was allowed between the injections sufficient for the production of a

TABLE 4.

Same animal that was employed in previous experiment—four days later. Urine in bladder gave no reduction, 2.0 grams urethane in 25 cc. water by mouth. Adrenalin employed, 1:250000.

TIME.	ADRENALIN INJECTED	URINE EXCRETED.	REDUCTION TEST.
	cc.	cc.	
10.46	0	—	—
11.05	34	10	negative
11.25	39	5	negative
11.45	38	4	negative
12.05	38	22	Strong
12.25	34	20	very strong

new store of glycogen. From an inspection of Table 5 it will be at once apparent that the intravenous administration of adrenalin in the dilution employed is far less potent in the non-narcotized rabbit than the usual subcutaneous injection with respect to the appearance of sugar in the urine. These results are in direct opposition to those of Ritzmann. The use of urethane in Ritzmann's experiments is undoubtedly the factor responsible for our failure to corroborate his conclusions. Our experiments also indicate the variation in the quantity of sugar eliminated by the normal rabbit maintained under constant conditions when equal doses of adrenalin are subcutaneously administered at different periods, an observation which has been corroborated for the dog.

TABLE 5.

RABBIT.	SUGAR IN URINE AFTER SUBCUTANEOUS INJECTION OF ONE MILLIGRAM ADRENALIN PER KILO IN DILUTION 1:1000		SUGAR IN URINE AFTER INTRAVENOUS INJECTION OF ONE MILLIGRAM ADRENALIN PER KILO IN DILUTION 1:125000.
	First injection	Second injection	
1	3.58	4.17	0.0
2	1.91	3.50	0.0
3	2.78	3.39	0.38
4	3.47	4.93	0.0

CONCLUSIONS.

Data are furnished from which it is concluded that adrenalin introduced in very dilute solutions (1:500000 to 1:125000) fails to induce glycosuria in the normal rabbit. On the other hand, when the animal is under the influence of urethane narcosis these dilute adrenalin solutions are a sufficient stimulus for the production of glycosuria.

From these observations it is apparent that urethane renders the rabbit organism unusually sensitive to the glycosuria-inducing action of adrenalin. The subcutaneous administration of adrenalin in the usual dilution (1:1000) to normal rabbits is far more efficacious in causing glycosuria than the same quantity of adrenalin introduced intravenously in much greater dilution.

The same quantity of adrenalin injected subcutaneously at different periods into the same animal under constant conditions causes the appearance in the urine of variable quantities of sugar.

THE SOURCES OF ERROR IN THE FOLIN METHOD FOR THE ESTIMATION OF CREATININE.

BY ALONZO ENGLEBERT TAYLOR.

(Received for publication, December 7, 1910.)

Having had occasion to direct and supervise the estimation of creatinine by the Folin method in over a thousand specimens of urine, certain sources of possible error in this excellent method have been brought forcibly to my attention. Certain of the errors are inherent in the method; others are due to misapplications of the method.

A standard dilution, in the estimation of normal urines, facilitates greatly the comparisons of results. In the normal cases with whose examinations I was associated, all urines were diluted to 2000 cc. Since in these cases the masses of creatinine ran from 1.5 to 2.0 grams daily the concentrations were near the center of the latitude permitted by Folin in the original description of the method. While it is true that from 5 to 15 milligrams of creatinine may be permitted in the volume of urine submitted to the test, it is clear that for comparative work a relatively large error is permitted in so wide a range of concentration. A constant dilution of the urine lessens this variable; a double estimation, in which the second deals with the amount of urine containing just 10 milligrams according to the first estimation, will obviate the error.

The varying intensity and qualities of normal and abnormal urinary pigments modify sometimes the values of the readings. Bile is quite disturbing, as are excessive degrees of the normal urochromes. Here again a standard dilution lessens the deflections. There are urines, however, in which an accurate estimation demands a decoloration of the urine. How this is to be accomplished, whether by mechanical or chemical means, would doubtless vary with the particular condition. Naturally it would in each instance need to be shown that the mass of creatine was not altered by the process of decoloration. Since so small a volume of urine is employed in a large volume of picric acid solution, the disturbing influence of the urinary pigments may be somewhat surprising. It is to be explained by the fact that under these circumstances a color distinction is added to a distinction of intensity, particularly confusing to the untrained eye.

To my mind, and to my eye, the greatest defect of the method lies in the absence of a standard light. Few normal eyes are perfect in colorimetric estimations, and not a few normal eyes are totally and permanently unfitted to such. This is not largely or primarily a question of color sense. As is well known to silk testers, all good judges of color are not good judges of shade. In the estimation of light and shadow in the polariscope, in work with the spectroscope, in the simple readings of the haemoglobinometer, similar observations are to be made; some individuals cannot make accurate or corresponding readings. Daylight is a varying factor, most of all in cities. One has to deal with high blue light, the brassy sky of the heated summer day, high white light, fog light, dark cloud light, the dull light of rainy days, at times with the addition of more or less smoke. With these different lights, it is often difficult to make readings with the colorimeter in which the range of minimal differences is not so wide as to lead obviously to gross errors. I have seen days of low fog in California in which an estimation of creatinine could not be made with less than a plus or minus error of 10 per cent. Gas light, the light of the carbon filament, the light of the tungsten filament, the light of the arc lamps are all unsuited to the estimation. It is possible that the light of the Moore tube might prove suitable. It is also possible that with some appropriate light filter, one or other of these above mentioned lights might prove satisfactory. But if the Folin method is to attain to that general usefulness and reliability to which its intrinsic qualities entitle it, a standard light must be found, standard in quality and intensity.

Under ideal conditions, the method will enable one to estimate 10 milligrams of creatinine with an accuracy of plus or minus 0.1 milligram. Under undesirable conditions of concentration, urinary pigmentation and light, the plus or minus error in the estimation may be at least a milligram. Accurate estimations and close agreements in the determinations of creatinine are greatly to be desired because in this case we deal with a fundamental metabolism, the full understanding of which we are just beginning to appreciate.

ON THE CUTANEOUS ELIMINATION OF NITROGEN, SULPHUR AND PHOSPHORUS.

By ALONZO ENGLEBERT TAYLOR.

(Received for publication, December 9, 1910.)

The present publication presents the results of two metabolic experiments, in which, for the purpose of securing normal data, the attempt was made to secure quantitative determinations of the magnitudes of the cutaneous eliminations of nitrogen, sulphur and phosphorus. I shall make no review of the literature, desiring merely to publish our results. The subjects of the tests were two healthy men of about forty and twenty-five years of age. They were technical assistants in laboratory work, and thus familiar with the controls demanded in metabolic experiments. The tests were carried out during the winter of 1910 in Berkeley, California. The men worked in laboratories not heated, in which the temperature during the period of investigation ranged from about 50° to 65° F. The men did each day, including Sundays, a uniform amount of work, at the quite uniform room temperature stated, and wore throughout the tests clothing of uniform weight. Conscious perspiration was not noted by either man during the course of the tests. The diet was of milk, cereal, bread and sugar, and was fixed by each man in a foreperiod and thus adapted to his habits and requirements; once thus fixed, it was constant during the time of the test. Obviously, what we desired to learn was the elimination of the named substances under normal uniform conditions, not the possible extent of such eliminations under excessive and uncontrolled conditions of work, external temperature and clothing. I have no doubt that under forced work and perspiration, much larger values than have been here noted could have been secured. We wished to form an idea of the data of normals, and of the range of individual variations.

The bodies of the men were washed daily with alcohol and 0.5 per cent solution of sodium carbonate. The underclothing and stockings were washed in a similar manner. The hair was not washed. Every attempt was made to make the washings quantitative. The wash waters were collected from day to day in large evaporating dishes, kept strongly acidulated with hydrochloric acid, and in the end the collections, representing the total secretions for the entire periods, were evaporated to nearly dryness. It was intended to carry the experiments through six weeks each. In the one case, however, at the end of four weeks the diet had to be discontinued on account of the impaction of the milk stools in the sigmoid flexure. The periods of tests were therefore 28 and 45 days.

The dessicated collections were first thoroughly extracted with absolute alcohol. The residue was then thoroughly extracted with water. The final residue, composed largely of coal soot, was dried. There were thus three fractions: the alcoholic extract, the watery extract and the insoluble residue. For the estimation of the total sulphur the materials were oxidised with sodium peroxide, and the SO_4 weighed as the barium salt, after precipitation according to Folin. For the estimation of the phosphorus, the materials were oxidised with magnesium oxide, according to the official method for the analysis of fertilizers, and the final estimation accomplished by the molybdate titration method. The nitrogen was determined by the Kjeldahl method. Since the fractions concerned in the analyses can have no importance, the totals alone will be given.

	EXPERIMENT J. 28 days.	EXPERIMENT D. 45 days.
	grams	*grams*
S	0.798	0.675
P	0.076	0.096
N	5.3	7.2

The average eliminations of S, P and N per day for the two men were therefore:

	gram	*gram*
S	0.028	0.015
P	0.003	0.002
N	0.190	0.160

These values are of course too low. Firstly, because the hair was not washed, and secondly, because there must have been some loss in the washings. When one recalls the richness of the secretions of the scalp, and contrasts the area of the scalp with that of the rest of the body, a certain approximation of this loss is permitted. It is quite certain to be larger for the sulphur than for the nitrogen. Probably 10 per cent would represent this loss for the nitrogen, and 20 per cent for the sulphur. The washings obviously include both elimination and desquamation. When one notes the minimal figures for the phosphorus, one is led to infer that in all likelihood this represents desquamation solely, being derived from the nuclei of the desquamated epithelial cells, and that therefore there is no real cutaneous elimination of phosphorus in any form. For the nitrogen eliminations there is a good agreement. For the sulphur eliminations however a wide individual variation is to be observed. In neither wash waters were body hairs to be seen in more than minimal numbers, so that the variation in sulphur cannot be attributed to this factor, important when one considers the sulphur content of hair. This variation is apparently then simply a variation in the cutaneous elimination of sulphur in two different men. As is well known, the skin in some individuals is a great deal more greasy then in others, and it is quite likely that this variation rests upon variation in the secretion of sulphurized lipoids.

Contrasting these figures with those of the metabolism, we have the following tabulations:

	EXPERIMENT J. 28 days.	EXPERIMENT D. 45 days.
Total S output	29.65	54.26
Urine S output	26.61	48.18
Faeces S output	2.24	5.40
Perspiration S output	0.79	0.67
Total P output	53.77	96.21
Urine P output	32.9	64.61
Faeces P output	20.79	31.50
Perspiration P output	0.07	0.09
Total N output	446.3	821.6
Urine N Output	413.0	737.9
Faeces N output	28.0	76.5
Perspiration N output	5.3	7.2

In all probability the cutaneous elimination of nitrogen and sulphur is, under constant conditions of work, clothing and temperature, a constant for each individual, and in no wise dependent on the total metabolism of nitrogen and sulphur in the individual. The cutaneous elimination of phosphorus may be said to be nil.

ON THE ESTIMATION OF UREA.

BY ALONZO ENGLEBERT TAYLOR.

(Received for publication, December 10, 1910.)

One encounters in the current literature of physiological chemistry certain misconceptions regarding the estimation of urea and the purposes of such determinations. These misconceptions are based upon the technical chemical criterion of quantitative analysis, in disregard of the physico-chemical law that the adaptability of a chemical method of analysis is determined not solely by the exactness of the method under the conditions of exact analysis, but by the conditions of the whole experiment and the control of the other variables in the experiment of which the particular chemical procedure constitutes one phase.

There are two methods for the estimation of urea concerned in this discussion, that of Folin and the first method of Benedict. The two methods give identical results with known solutions of urea. Applied to the urine the method of Benedict gives slightly higher results; apparently that of Folin when carefully executed gives very accurate results. The slightly higher results of the method of Benedict are due to the fact that a small amount of uric acid and creatinine are hydrolyzed to urea. This may be minimized by having the urine dilute, by restricting the amount of hydrochloric acid to but little more than the amount necessary to combine with the ammonia evolved from the urea, and limiting the pressure of the autoclave to five atmospheres and the time of the heating to one hour. Under these conditions, the Benedict method will yield figures that are about one per cent higher than those furnished by the Folin method. The defects of the Folin method are that the reaction must be carefully watched during its first stage, and that when the ammonia is distilled off, this distillation must be carried practically to the point of dryness of the residue. When they are to be done in large numbers, the estimations by the method of Benedict are much more easily done than are a similar number of determinations with the method of Folin.

When one desires to learn exactly the amount of urea in a urine, the method of Folin is the one to be employed. When in a metabolic experiment in which the lower limit of protein input is being sought, the absolute output of urea is being determined, the method of Folin should be employed. On the contrary it can be easily shown that in a metabolic experiment dealing with a subject on a natural, known and fixed diet, where the nitrogen balance is being determined and the partition of nitrogen into the terms of urea-nitrogen, creatinine-nitrogen, purine-nitrogen, ammonia-nitrogen, hippuric-nitrogen and rest-nitrogen is being defined, the method of Benedict fulfills every physiological and physico-chemical requirement.

For what is the rôle of the figure for the urea-nitrogen in such a balance and partition experiment? The ratio of the urea-nitrogen to the total nitrogen is merely a function of the nitrogen input, since the body resists all storage of nitrogen and the excess of protein input is hydrolyzed and eliminated as urea. While it is probably true that if the exogenous creatinine be excluded, the elimination of creatinine is slightly higher on a rich protein input, as a matter of approximate fact it is certain that the creatinine-nitrogen, the purine-nitrogen, and the rest-nitrogen are expressions of metabolic entities, and are quite uninfluenced by the excess of simple protein in the diet. The relations of the ammonia-nitrogen are not clear, but this fact has little relevancy to the matter under discussion. The urea-nitrogen represents the sums of probably three functions; an essential urea output, corresponding to the essential purine output for illustration, a fraction which according to the investigations of Folin must be small; a fraction due to the conversion of uric acid and creatinine into urea, a fraction of which the extent is undetermined though small; and the larger fraction representing simply the output corresponding to the excess of protein in the diet. Now the exact estimation of the urea in a balance experiment simply means that the urea percentage of the total nitrogen will be found a little lower than if the determination were done by the Benedict method. This slightly lower percentage has no meaning in interpretation since the normal variations and fluctuations noted in different individuals and indeed at different times in the same individual are greater than the increment over the values determined by the Folin method that are yielded by the Benedict method. So far as the partition figures are concerned, the fact simply is that when the Benedict method is used instead of the Folin method, the figure for urea is about 1 per cent higher and the figure for rest-nitrogen about 1 per cent lower. Since within these limits no interpretation can be placed on the exact estimation of the rest-nitrogen, the results obtained by the Folin method mean no more and nothing else than those determined by the Benedict method. The partitions that are of value for accurate determinations are those of creatinine, purine, ammonia and hippuric acid in some cases. The value of the urea is solely in relation to the total nitrogen, and for this purpose whether the percentage be exact or 1 per cent too high, has little meaning. The real questions are two: Is the individual in nitrogen balance? What are the amounts of ammonia, creatinine, purin and possibly hippuric acid? What is the amount of rest-nitrogen? For the determination of the last, we need an estimation of urea that shall be fairly accurate, simply to show that the rest-nitrogen of which little is known, lies within the rather wide normal limits for this fraction. For such an estimation of urea the older methods, of which the hypobromite may be taken as a type, are of course too grossly inaccurate. But the method of Benedict, that varies in its results always in the one direction of increase, and of which the degree of increase can always be held within narrow limits, is fully competent. It is here as elsewhere, we must adapt our method to our problem. There is no use in employing the most accurate method for the estimation of the chlorides of the urine, since a large amount of chloride goes out by the skin and the urinary elimination is secondary thereto.

There are many investigations in which metabolic studies are employed partly for the direct information they yield, and partly as adjuncts. For many experiments it is necessary to know that the body is in balance, it is necessary to know accurately the amounts of creatinine, purine and ammonia; and the amounts of urea and rest-nitrogen in a fairly accurate manner. For many such investigations, abnormal findings in these directions will not be found and are not to be expected. But the figures for the metabolic experiment, in themselves normal, give to the experimenter a guarantee of control in the experiment that enables him to trust any abnormality that may be detected in any special function. For such investigations, the Benedict method, much the easier of execution in large numbers, is quite as good as the method of Folin. Extreme exactness in an estimation is to be sought only when the interpretation is directly proportional to the accuracy of the measurement of the variable, and when in the other conditions of the experiment there are no uncontrolled variables of larger magnitude than that of the analysis in question. In a certain metabolic experiment the following values for the period of seven days were determined:

	Total nitrogen; 82.6 grams			*If urea by Folin, about*
	grams		*per cent.*	*per cent.*
Urea-nitrogen	72.8	=	88.1	87.1
Creatinine-nitrogen	3.1	=	3.7	3.7
Purine-nitrogen	1.4	=	1.7	1.7
Ammonia-nitrogen	1.8	=	2.1	2.1
Rest-nitrogen	3.5	=	4.2	5.2

The subject was in nitrogen balance. The urea values were determined by the method of Benedict. Had these figures for urea been determined by the method of Folin, the percentage for urea-nitrogen would have been slightly lower than those in the table, probably one per cent lower, and the values for the rest-nitrogen would have been that much higher. Can any one believe that the interpretation of the one set of figures would be anything but identical with the interpretation of the other set? The two sets of figures have of course the same and identical value. When the day comes on which a fluctation of one per cent of the total nitrogen (of an ordinary diet) between the urea-nitrogen and the rest-nitrogen has a meaning and can be used as a basis for a physiological or pathological interpretation, then the day will have come for the teleological employment of the more laborious but exact method of Folin instead of the rapid, easy and fairly accurate method of Benedict. The new method of Benedict I have not yet tried. From the descriptions, the increased accuracy will be paid for in time and attention. There is no amount of time and attention too great to devote to a method of analysis when the correct definition of the problem demands it; but there is no use in calculating results to the third place in decimals when the interpretation cannot go beyond the first place.

THE COMPOSITION OF INVERTASE.

BY ALBERT P. MATHEWS AND T. H. GLENN.

(From the Laboratory of Bio-Chemistry and Pharmacology, the University of Chicago.)

(Received for publication, December 16, 1910.)

For a study of the chemical composition of ferments, invertase has many points of advantage over its near relative diastase. Invertase acts by a single, simple reaction on a crystalline substance, whereas diastase is most probably a mixture of amylase, and possibly various dextrinases which by a series of transformations produce maltose.

The best and most complete investigation of the nature of invertase is that of O'Sullivan and Tompson[1] in 1890. These observers took advantage of the fact that yeast, when allowed to stand, digests itself and changes in the course of two to six weeks to a brownish yellow liquid and an insoluble residue. This liquid, which changes its composition very little even when kept for months, has a strong inverting action and was used for their study of the properties of invertase. These authors discovered the fundamental facts of the enormous acceleration of the action of the ferment by a very minute amount of acid and the stopping of its action and destruction of the ferment by alkali. They determined the temperature coefficient as about 2 for each 10 degrees of temperature up to 35° C. and at less than 2 above this point; they, for the first time, showed how to measure accurately the strength of any powder called invertase and thus found a method of establishing the activity of the substances isolated. This enabled them to isolate and study invertase. They showed that the invertase inverted cane sugar in such a way that the velocity of the inversion as measured by an equation of a reaction of the first order was proportional to the amount of ferment added; and that the disagreement of the observed inversion, as seen in the polariscope, from the computed rate was only apparent and not real and was due to the multirotation of the glucose. By means of the addition of alkali this multirotation could be hastened and the real velocity of inversion directly measured in the polariscope. A knowledge of O'Sullivan and Tompson's paper would have saved the mistaken conclusions of Henri[1] and other investigators, who did not allow for the multirotation of the glucose, and hence concluded that the reaction of invertase on cane sugar did not follow the equation of the simple reaction of hydrochloric acid on this sugar. Hudson[2] has recently repeated and confirmed a part of O'Sullivan and Thompson's work and has extended it in some particulars.

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[1] O'Sullivan and Tompson: *Journal of the Chemical Society*, lvii, p. 834, 1890.

O'Sullivan and Tompson attempted to purify the ferment by repeated precipitation by alcohol of 47.5 per cent and resolution of the ferment in water and dialyzing to remove the salts, mainly phosphates, which constituted nearly 30 per cent of the first raw product. They found that the ferment lost strength with every precipitation, and that when it was most pure it was easily destroyed by dialysis and precipitation with alcohol. They found that when the salts were completely, or nearly completely, dialyzed out that the addition of alcohol would no longer flock the ferment, but produced a milky solution which would not precipitate. To precipitate it they had to add some acid and when thus precipitated by alcohol and acid the precipitate was entirely inactive.

Their purest and most active preparation would not dialyze and was hence a colloid. Its molecular weight was at least 1885 and probably higher. It was strongly dextro-rotatory, $[\alpha_j^{20°}] = +50.56°$. The most powerful preparation was of such power that an amount containing one gram of organic matter when dissolved with 100 grams cane sugar in 500 cc. of water with optimum strength of sulphuric acid at 15.5° inverted 74.1 per cent of the cane sugar in 25.1 minutes. As a rule the preparations were a little weaker and required 28–30 minutes. 74.1 per cent inversion yields a mixture of cane sugar and invert sugar which is optically inactive ($a = \pm 0$). This still contained 15.3 per cent ash consisting mostly of calcium and magnesium phosphates. By dialysis they reduced the ash to 2.4 per cent and finally to 0.45 per cent without any reduction of the activity of the organic matter present and they concluded from this that the ash is not an essential part of the ferment. They could not, however, recover this pure invertase by precipitation for the reasons already cited. It lost its activity on precipitation with acid alcohol. The ash-containing powder gave a strong Millon and xanthoproteic reaction, but no biuret. This powder ("B"), although very active, was not so active as before precipitation. It analyzed, ash-free, C, 46.5; H, 6.91; N, 6.2 per cent.

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[1] Henri: *Lois générales de l'action des diastases*, Paris, 1903, p. 55.

[2] Hudson: *Journal of the American Chemical Society*, xxx, p. 1160, 1908; *Ibid.*, p. 1564.

They studied the chemical composition of the inactive substances obtained by acid alcohol precipitation. One of these products was insoluble in cold water and contained nitrogen. They called it yeast albuminoid. It contained C, 54.27; H, 7.54; N, 14.07 per cent. It gave a strong Millon, but no biuret reaction. The inactive precipitates obtained by acid alcohol precipitation contained varying amounts of nitrogen. A series of bodies were thus obtained called invertans. They analyzed as follows:

		H	N	ACTIVITY
Yeast albuminoid	54.06	7.35	14.53	Inactive
α-Invertan	48.03	6.65	8.35	"
β-Invertan................	46.41	6.63	3.69	Active Invertase
γ-Invertan	45.62	6.55	3.15	Inactive
δ-Invertan.	46.50	6.82	2.43	"
ε-Invertan................	44.45	6.36	2.07	"
ζ-Invertan................	44.73	6.40	1.61	"
η-Invertan................			1.05	"

ε-Invertan gave a large amount of a reducing sugar on boiling with acid and we now know that this sugar is largely mannose. They concluded that invertase proper is a union of yeast albuminoid and η-invertan, which is largely carbohydrate. These substances are inactive alone.

These observers found, also, that the presence of cane sugar decidedly protects invertase from the action of heat. Thus with no sugar present the ferment was completely destroyed by heating to 55°, and 70 per cent destroyed when heated to 45°; but with 20 per cent of cane sugar present no destruction took place until 60° was reached; at 65°, 12 per cent was destroyed; at 70°, 66 per cent; at 75°, 100 per cent. They rightly interpret these facts as showing that the ferment combines with the cane sugar. A similar protective action toward invertase has been shown by Jodlbauer[1] to exist also in the case of glucose, levulose, maltose, glycerin and other carbohydrates, thus showing that invertase combines with a great many polyatomic alcohols and it possibly combines also with ethyl alcohol.

O'Sullivan and Tompson's conclusions, therefore, were that invertase is a compound of an albuminoid with a carbohydrate.

Salkowski[2] in 1900 showed that invertase, as prepared from yeast, always contained yeast gum, or mannosan, which yields a large quantity of mannose on heating with acid. This had been previously discovered by Hessenland[3] in the invertase from the sugar beet, which also yields a large quantity of d-mannose on heating with acid. Salkowski concluded that this mannosan gum was present only as an impurity in the invertase and that it was not part of the ferment. O'Sullivan and Tompson's γ- and ϵ-invertan were undoubtedly, he thought, composed chiefly of this mannosan. Salkowski[4] was equally certain, since the invertase gave no protein tests except a very weak tyrosin and xanthoproteic, that it was not a protein, whatever else it was. He got an odor of pyrrol by heating in a tube a portion of his ferment. His analysis of the invertase was C, 53.94; H, 8.56; N, 8.41. Since the yeast gum, or mannosan, yields C, 42.41; H, 6.43, this leaves for the nitrogenous constituent, in one case, C, 59.99; H, 12.54; N, 16.86; and in another case, C, 56.40; H, 8.99; N, 10.15. We shall show that there is no incompatibility of O'Sullivan and Tompson's and Salkowski's results.

Wroblewski[5] in 1901 studied invertase in Buchner's press juice. He found that invertase could not be salted out of the preparation by ammonium sulphate and it was not precipitated by acetic acid. It was not destroyed by trypsin. It was destroyed by weak alkalies; its action was retarded by alkaline and hastened by acid phosphates. By long action of water alone it was weakened. Contrary to O'Sullivan and Tompson, he found that invertase dialyzes, but with extreme slowness. He concludes, although he offers no evidence for his conclusion except analogy with his results on diastase and earlier work in which a small amount of invertase was salted out and found to be protein,[1] that invertase is certainly a protein and must be related either to peptone or to a secondary albumose since it is not salted out.[2] He gives no figures by which the strength of his invertase can be directly compared with that of other men, since he allowed the invertase to act for an hour, in a very concentrated solution at 37° C., by which time nearly all the cane sugar had been inverted and slight errors in reading the polariscope would introduce an enormous error into the computation of the velocity constant. It is, however, certain that Wroblewski had a good strong invertase. He regarded the carbohydrate present to be an impurity and the ferment itself a protein.

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[1] Jodlbauer: *Biochemische Zeitschrift*, iii, p. 488, 1907.

[2] Salkowski: *Zeitschrift für physiologische Chemie*, xxxi, p. 305, 1900.

[3] Hessenland: *Zeitschrift des Vereins f. die Rübenzuckerind.*, xlii, p. 671, 1892.

[4] Salkowski: *Ibid.*, p. 315.

[5] Wroblewski: *Journal für praktische Chemie*, lxiv, p. 1, 1901.

Osborne[3] and Kölle[4] working under Hüfner's direction prepared invertase from yeast by first coagulating the protein by alcohol. The alcohol was decanted off and chloroform water was added to the residue. This was allowed to stand for six days at 30° C.–35° C. The mixture was then filtered until the filtrate became clear. The yellowish solution was precipitated with alcohol, washed with absolute alcohol, and dried. The preparation obtained contained from 31–40 per cent ash.

To remove the ash, Osborne tried several methods. The most successful one was to dissolve the preparation obtained by the above method in distilled water, add ammonium-magnesium mixture to precipitate the phosphates and to remove the excess of the reagent by dialysis. By this method a preparation was obtained which contained very little ash, but which still contained 6 per cent of nitrogen. He concluded that invertase is a carbohydrate containting nitrogen and resembles chitin. His preparation contained quite regularly about 44.69 C; 6.51 H; 6.10 N; and 1.7–8 per cent of ash. The substance gave weak biuret, Millon, and xanthoproteic reactions. From his results, however, it was not possible to decide whether the small amount of protein was the active principle or whether it was present as an impurity. He gives no figures showing the quantitative inverting power of his purified invertase. The nature of the carbohydrate split off was investigated by Kölle, who obtained an invertase, using Osborne's method, of the following composition; ash 3.96–10.86 per cent. Computed ash-free: C, 43.90–45.65; H, 6.45–7.34; N, 8.32–8.46. The activity is only given qualitatively. This substance yielded mannose in large quantities. Michaelis[1] in 1907 showed that invertase was probably an electro-negative colloid, since it was precipitated by colloidal ferric hydrate and lead oxide, but not by kaolin or electro-negative colloids. His conclusions, however, are not worth a great deal in our opinion since he used the very crude material made by rubbing yeast with chloroform and sand; and it is possible the ferric hydrate combined with some colloid and with the ferment too, all going down together. Jodlbauer[2] has shown that invertase probably combines with d-glucose, levulose, mannose, d-galactose, lactose, and maltose, since these substances protect it from the destructive action of ultra-violet light.

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[1] Wroblewski: *Berichte d. deutsch. chem. Gesellschaft*, xxxi, p. 1134, 1898.

[2] Wroblewski states in one paper that invertase was salted out by ammonium sulphate and in the other that it was not.

[3] Osborne, W. A.: *Zeitschrift für physiologische Chemie*, xxviii, p. 399, 1899.

[4] Kölle: *Ibid.*, xxix, p. 429, 1900.

In the following table we have recapitulated the results which have been obtained in the chemical examination of invertase.

	C	H	N	ASH
Barth*	44.44	8.4	5.95	22
O'Sullivan-Tompson	46.41	6.63	3.69	
	46.50	6.91	6.20†	
Osborne	44.69	6.51	6.10	
Kölle	43.90–45.65	6.45–7.34	8.32–8.46	4–10
Salkowski	(1) 53.94	8.56	8.41	
	(2) 48.31	8.58	5.96	

* Barth: Ber. d. deutsch. chem. Gesellsch., xi, p. 474, 1878.
† Analysis of precipitated powder, "B."

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[1] Michaelis: *Biochemische Zeitschrift*, vii, p. 488. 1907.
[2] Jodlbauer: *loc. cit.*

From this review it is clear that no unanimity of opinion exists concerning the nature of invertase, it being regarded as a protein, a carbohydrate, a glucoside, and as something other than any of these.

1. METHOD.

Thirty pounds of commercial compressed yeast were placed in a number of flasks and sufficient water added to moisten it. Toluol was added to prevent bacterial growth. The flasks were allowed to stand for four weeks at room temperature. Strong fermentation takes place and liquefaction. At the end of this time, a brownish liquid has collected over a yellowish residue. The dirty brown liquid (tyrosinase?) obtained was filtered through coarse filter paper to remove all undissolved particles. The filtrate was then decolorized with charcoal and filtered twice through infusorial earth in a Buchner funnel.

The filtrate was yellowish in color, but perfectly clear, readily inverted cane sugar, but gave a fairly strong Millon test and a faint biuret reaction. Toluol was added to the solution and it was allowed to stand in the ice box over night. In the morning tyrosin-like crystals, giving a Millon reaction, had crystallized out. These were removed and the liquid was placed in the cold at about 0° C. for 12 hours. The liquid became slightly cloudy and a very small quantity of crystals, supposed to be tyrosin from the Millon test, formed on the bottom of the flask. The solution was filtered while cold to remove tyrosin and again filtered through infusorial earth in an Buchner funnel until perfectly clear. The clear yellow filtrate was precipitated with 5 volumes of 95 per cent alcohol. A white flocculent precipitate came down which gathered in balls and stuck to the sides of the beaker. The alcohol was decanted off and the sticky mass was treated with distilled water. The portion of the substance not easily dissolved (phosphates of calcium) was filtered off and the water extract again precipitated with alcohol. The flocculent, less sticky precipitate was again dissolved in distilled water, the less soluble portion removed and again precipitated with alcohol. This process was repeated five times in all. The substance obtained from the fifth precipitate was filtered off, washed with absolute alcohol, and dried in a vacuum desiccator.

The preparation obtained readily inverted cane sugar, dissolved easily in distilled water and gave very faint protein reactions, some of which were at first overlooked they were so faint. The Millon and xanthoproteic were the strongest, but still faint unless a good deal of material was taken for the test. The biuret test was at first recorded as negative. A good Molisch test for carbohydrate was obtained; the preparation still contained much ash, mostly in the form of phosphates.

To remove the ash, Osborne's method[1] was resorted to. The white substance was dissolved in a small amount of distilled water, ammonium magnesium mixture was added until no more substance was precipitated. The precipitate was filtered off, suction being used, and the filtrate was placed in parchment sacks and allowed to dialyze in running water for five days. The solution was precipitated with four volumes of 95 per cent alcohol. It precipitated very slowly. It was washed with absolute alcohol and dried in a vacuum. The preparation thus obtained was a snow-white amorphous powder, very active when a trace of acid was added to it. The biuret test was at first recorded as negative, the very faint biuret test being overlooked. Millon and xanthoproteic were extremely faint. The substance was thought to contain, therefore, no protein but a small tyrosin impurity. Our first conclusion was that to which Salkowski had already come. The powder dissolved readily in water to an opalescent solution slightly tinged with yellow in a strong solution. It was insoluble in alcohol and ether. It contained little ash and this was chiefly magnesium phosphate. It did not reduce Fehling's solution. The analysis gave the following results:

0.2014 gram invertase burned gave .0024 gram ash = 1.1 per cent.

0.2789 gram invertase by Kjeldahl gave ammonia neutralizing 3.3 cc. $\frac{N}{10}$ H_2SO_4 = 1.67 per cent N, ash free.

0.2693 gram invertase = 3 cc. $\frac{N}{10}$ H_2SO_4 = 1.56 per cent N.

0.4061 gram invertase = 3.5 cc. standard acid. 1 cc. = .002 gm. N = 1.74 per cent N.

Mean N = 1.66 per cent.

0.2097 gram boiled 3 hours with 2 per cent HCl gave by Munson and Walker's method 0.2110 gram Cu_2O = .0942 gram mannose = 45.4 per cent carbohydrate, computed ash free.

0.249 gram invertase heated 30 hours with 2 per cent HCl gave 0.3658 gram Cu_2O = .1705 glucose = 70 per cent.

0.2638 gram invertase = 0.0026 gram ash = 0.98 per cent ash.

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[1] Osborne: *loc. cit.*

We thus obtained a preparation of invertase which was active and which contains less ash, *i.e.*, 1.0 per cent, and less nitrogen, 1.66 per cent, than any active preparation yet obtained and consists of 70 per cent of its weight of a reducing sugar. The nitrogen is about one-fourth that of Osborne; about one-fifth that of Kölle; about one-fourth to one-fifth that of Salkowski; and about one-fourth that of O'Sullivan and Tompsons' preparation "B" and a little less than one-third their most active invertase. Our figures for ash and nitrogen are like those given by O'Sullivan and Tompson for their ζ-invertan, but their preparation was entirely inactive. The almost total absence of all albumin tests, except a faint Millon, indicated that the ferment was not a protein and we at first concluded that the ferment was probably a carbohydrate, containing some tyrosin as an impurity, agreeing in this respect with Salkowski. As will be seen, later investigation changed this opinion.

2. ACTIVITY.

Having thus obtained an active invertase consisting to the extent of at least 70 per cent of its weight of a mannosan or other gum and with a very low nitrogen and ash, the question arose whether our purified preparation was more or less active than those which had been obtained by others. We have evidently eliminated in the course of preparation most of the nitrogenous-constituents of the ferment and correspondingly increased the non-nitrogenous residue. If the active principle is the nitrogen-containing part, our preparation should be decidedly less active than any similarly purified hitherto reported; if the active principle is the carbohydrate portion our preparation should be at least as active as that of others, providing other changes have not come in. The activity of our preparation was measured by dissolving varying amounts of the dry powder in 10 cc. of a 10 per cent cane sugar solution previously warmed to 35° C. and allowing the solution to stand one hour at 35° C. At the end of that time the solution was quickly heated to boiling and the invert sugar determined by the reduction of an equal quantity of Fehling's solution, the cuprous oxide filtered through an asbestos Gooch crucible and weighed after washing with alcohol and ether according to the method of Munson and Walker.[1] The results are given in Table I.

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[1] Munson and Walker: *Journal of the American Chemical Society*, xxviii, p. 663, 1906.

TABLE I.

No acid added. Showing activity of invertase on cane sugar.

EXP.	10% CANE SUGAR	INVERTASE	TIME	TEMP.	AMT. OF CU_2O	CORRESPONDING AMT. OF INVERT SUGAR
	cc.	*mg.*	*hours*		*mg.*	*mg.*
1	10	0	1	35° C.	0	0
2	10	0.5	1	35° C.	28	12.5
3	10	1.3	1	35° C.	100	45.0
4	10	2.6	1	35° C.	197	90.6
5	10	5.2	1	35° C.	328	155.8
6	10	13.0	1	35° C.	947	468.0
7	10	1.3	3	35° C.	250	116.4

Small quantities of weak acid accelerate the activity of the enzyme strongly as Table II shows:

TABLE II.

EXP.	10% CANE SUGAR	ACETIC ACID 10%	TIME	TEMP.	INVERTASE	CU_2O	CORRESPONDING AMT. OF INVERT SUGAR
	cc.	*cc.*	*hours*		*mg.*	*mg.*	*mg.*
1	10	0.2	1	35° C.	0	0	0
2	10	0.2	1	35° C.	0.6	38.89	16.9
3	10	0.2	1	35° C.	1.5	160.0	73.0
4	10	0	1	35° C.	0.6	32.0	14.3
5	10	0	1	35° C.	1.5	112.0	50.6

To measure the activity of the ferment the constant, K, was computed from these figures using the mono-molecular formula:

$$\frac{dx}{dt} = K \times C_{\text{(invertase)}} \times C_{\text{(cane sugar)}}$$

If $A = C$ (cane sugar at the start), $A - x =$ cane sugar at time t $\frac{dx}{dt} = K \times C_{\text{(inv.)}} \times (A-x)$. $C_{\text{(inv.)}} =$ grams invertase in 100 cc. of solution. $\frac{1}{C_{\text{(invertase)}} \times t} \log. \frac{A}{A-x} = K$

By this formula K was computed when C (invertase) was expressed in grams in 100 cc., and the slight reduction by the sucrose was disregarded.

EXP.	PER CENT INVERTASE	*t*	*K*
		min.	
1	.005	60	.0175
2	.013	60	.0256
3	.026	60	.0264
4	.052	60	.0236
5	.013	180	.0221
6	.006	60	.0174
7	.015	60	.0251
8	.13	60	.0385
9	.015	60	.0366 { with acid

Mean *K*, omitting 1, 6, 8 and 9, = .0244

It will be seen that a fair constant is obtained if we disregard the figures where only 0.5 – 0.6 mg. of invertase were used in which errors of weighing are relatively large, and No. 8 where too much cuprous oxide was found. The first figures are probably in error; for the last the computation of the amount of glucose was uncertain as the cuprous oxide obtained was twice that of the highest figures given in Munson and Walker's table, and the corresponding amount of invert sugar was uncertain.

To compare the activity of this invertase with the crude product of Osborne, since he gives no figures for his purified product, we have computed *K* from his results (p. 410) in which he inverted cane sugar at the same temperature. A 0.091 per cent solution of his crude product A inverted 86.2 per cent of the cane sugar in 30 minutes. Hence

$$\frac{1}{.091 \times 30} \log \frac{1}{.138} = K.$$

$K = 0.3107$ for preparation A;
0.4950 for B; and 0.0813 for preparation C.

His crude products, therefore, were much more powerful than our pure product although his method of determining the inversion, *i.e.*, by titration of small quantities with Fehling's solution until no color remains is of course quite inaccurate. His pure preparations were probably less active than these crude ones, although he gives no quantitative data concerning them. As his preparation B is much more active even than those of O'Sullivan and

Tompson and his method of determining the amount of invert sugar is inaccurate, a very small error in the Fehling titration causing a great change in the constant, not much weight can be laid upon these results. We may, therefore, throw out the result of preparation B as probably an error. We then have left preparation A, which is thus about twelve times as powerful as our figures, and preparation C which is about three times.

Our preparation was, however, slightly alkaline while his was slightly acid and the comparisons to be correct should be made only at the optimum acidity. Table II shows in experiment 9 the effect of an addition of acetic acid to our preparation. In this case K increased to 0.0366. Unfortunately we did not carry out any experiments upon the effects of small amounts of acid except those mentioned until nearly a year had passed.[1] The invertase preparation had stood as a dry powder in a vial protected from light during this time. According to O'Sullivan and Tompson it should lose some of its activity under these conditions. We nevertheless took this preparation and added small amounts of hydrochloric acid to it to get the velocity in an optimum concentration of acid, but we unfortunately used up the small amount of material at that time in our hands before we reached the optimum concentration of acid, although we were probably not far from it judging from O'Sullivan and Tompson's and Hudson's figures and our later results. Table III gives the results we obtained at 20.5.°

From the value of K in Table III it is seen that the addition of hydrochloric acid sufficient to make the solution .0003 N, more than trebles the rate of inversion and that 0.0001 N hydrochloric acid more than doubles it. If the temperature coefficient of the reaction is only 2 for each 10 degrees as shown by O'Sullivan and Tompson, the velocity of inversion of IV at 35° would be: $K_{35°} = 0.0839$.

Our preparation, therefore, is certainly, as active as Osborne's weaker one C and about one-fourth to one-third as active as his crude product A.

The comparison of our preparation with O'Sullivan and Tompson is more satisfactory since they used all precautions

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[1] This was owing to the fact that the work was begun by Mr. Glenn at my suggestion, and continued by me after the lapse of a year. A. P. M.

TABLE III.

	I	II	III	IV
Concentration HCl.....	0	.0001N	.0002N	.0003N
Concentration cane sugar...............	5%	5%	5%	5%
Concentration invertase	.025%	.025%	.025%	.025%
Reading at start 2 dm. tube 20.5°..........	6.92°	6.92°	6.92°	6.92°
Reading after 30 minutes................	6.87°	6.70°		
Reading after 35 minutes			6.60	6.43
Reading after 1 hour and addition of Na_2CO_3..............	6.66^0	6.36°	6.22°	6.05°
K =.................	.00844	0.018	0.0233	0.0289

$$\frac{1}{\text{per cent invert} \times t} \log_{10} \frac{a_0 - a_\infty}{a_t - a_\infty} = K_{35°} = .0839$$

to secure an optimum acidity. In their case, 74.1 per cent of the cane sugar was transformed in 26 minutes at 15.5° by the most active preparation, or in an average of 30 minutes by the slightly less active. Computing K from their figures of the concentration of the organic matter of the majority of their preparations, we have

$$\frac{1}{.2 \times 30} \log \frac{1}{.259} = K . \quad K = .0978$$

at 15.5° or 0.1467 at 20.5° or 0.3912 at 35°. Their invertase is, therefore, from four to five times as powerful as ours.

The foregoing comparison shows then that our preparation was decidedly lower in its fermentative power than either O'Sullivan and Tompson's or Osborne's crude substance. It was clear, therefore, that with the purification of our preparation, a process which presumably had reduced the nitrogen, had also resulted in a loss of activity. This led us to change our opinion that the active principle was the carbohydrate part of the molecule and the nitrogen was in a tyrosin impurity, and we concluded it to be more probable that the active principle, whatever its nature, was in the nitrogen part of the molecule.

3. DOES INVERTASE GIVE THE PROTEIN REACTIONS?

Having obtained the result that our preparation was not only poor in nitrogen, but also equally poor in its activity, we turned back and examined the invertase with great care to see if active preparations could be obtained which did not give the biuret test. All of our preparations and those reported by others had given a faint but positive Millon and xanthoproteic reactions. Several preparations have been examined containing 1.5–1.6 per cent of nitrogen and of the same activity as our first preparation, which we thought was biuret free. We found that if a considerable amount of the powder was taken, a dilute solution of copper sulphate used and the preparation not heated, but allowed to stand for from 24–72 hours at room temperature, that always the blue precipitate of the copper-gum compound took on a faint lilac, which could be detected by comparison with the similar precipitate of the purified nitrogen-free gum. Since the solution does not contain the lilac colored substance but the color is on the precipitate, the color is concealed and will be overlooked if the preparation is boiled or if it is not allowed to stand for several hours or even days. *We have never found any preparation which failed in giving a very faint biuret if examined in this way, as long as it remained active* and all preparations give positive Millon and xanthoproteic reactions. Since the test is so easily overlooked, we are of the opinion that preparations reported as biuret free, but Millon positive, including our own early preparations, would probably give the biuret reaction if the test is carefully made in this manner. The biuret test is also less delicate than the Millon, as it is ordinarily performed. The sulphur test with lead acetate was always negative. The Adamkiewicz reaction either with glyoxylic acid or glacial acetic never gave a positive test that we could be sure of. Sometimes it seemed in strong solutions as if a very faint and evanescent violet appeared for a moment, but we are not sure of it.

We conclude then, that *all active preparations give the characteristic protein tests, i.e., biuret, Millon, and xanthoproteic, and that all of them contain a protein residue.* This confirms Wroblewski's[1] view.

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[1] Wroblewski: *Berichte d. deutsch. chem. Gesellschaft,* xxxi, p. 1134, 1898.

Fraenkel and Hamburg[1] have reported that they have a diastase which gives no protein tests other than at times a weak Millon reaction. In view of the facts above stated, the positive observations of Osborne[2] and Wroblewski[3] showing diastase to be a protein and the remarkable resemblance between diastase and invertase, we consider it possible that they may have overlooked the faint biuret reaction, and their diastase may not have been protein-free.

The nitrogen-containing substance in the invertase is hence a protein, but more complex probably than the figures obtained by O'Sullivan and Tompson for yeast albuminoid would indicate. They thought their yeast albuminoid had a molecular weight of about 190, and a formula corresponding to an amino-tyrosin.

4. A FURTHER COMPARISON OF THE NITROGEN CONTENT AND ACTIVITY.

We next examined carefully the relation between nitrogen content and activity and the influence of precipitation by alcohol.

Several lots of self-digested yeast have been examined in this way. The method of preparation was essentially that used at first except that the precipitation by ammonium-magnesium phosphate was omitted.

(a) *The original filtrate.*

Five cc. of the crude but clear and colorless original filtrate dried on the water bath and then at 110° for 1 hour contained 0.5157 gram of solids, which consisted of 0.4491 gram of organic solids and 0.0666 gram of ash. The solids, therefore, contained a little less than 13 per cent of ash. By Kjeldahl determination the organic matter gave 0.0447 gram N or 9.95 per cent. The activity was measured by adding 5 cc. to 100 cc. 10 per cent cane sugar containing 0.6 cc. $\frac{N}{10}$ H_2SO_4, which gave the optimum acidity. At 20° $K = 0.00523$ computed on the organic matter present. Two other lots gave essentially the same result. By Kjeldahl determination the N in the crude organic matter was 9.75 and 9.44 per cent and the constant was K (20°) = 0.00618 and 0.00689.

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[1] Fraenkel and Hamburg: Hofmeister's *Beiträge zur chem. Physiologie,* viii, p. 389, 1906.

[2] Osborne, T. B.: *Journal of the American Chemical Society,* xvii, p. 587, 1895. *Ibid.,* xviii, p. 1, 1896. *Berichte d. deutschen chem. Gesellschaft,* xxxi, p. 254, 1898.

[3] Wroblewski: *Ber. d. deutsch. chem. Gesellsch.,* xxxi, p. 1128, 1898.

The crude filtrate with the activity just stated was precipitated by adding alcohol to 50 per cent by weight. The precipitated gum was washed by successive portions of 60 per cent, 70 per cent, 95 per cent and absolute alcohol and ether, the greyish white gum transferred to watch glasses and dried over calcium chloride in vacuum desiccators frequently pumped out for several days to get rid of ether and alcohol.

The gum thus purified was powdered, dried at 105° to approximately constant weight for 4 hours and its water content, ash and nitrogen determined. The activity of the unheated gum was also measured. The two gum preparations from different lots of yeast analyzed above now contained respectively 4.08 per cent N and 5.00 per cent N and activities $K_{20^\circ} = 0.02843$ and $K_{20^\circ} = 0.0215$ computed on the organic matter. By this first precipitation, therefore, we had reduced the nitrogenous impurities. The nitrogen fell from 9.5 to 4.5 per cent in the average and the activity rose from 0.0065 to 0.025 on the average. The first precipitation evidently gave a partial purification. The powder still gave the protein tests, but less intense than before.

By re-solution, filtering from the undissolved phosphates and reprecipitation with alcohol, using where necessary a small amount of calcium chloride to flock the precipitate, two white powders were obtained, one, IV B (1) and the other, IV A (3). Preparation IV B (1) resembled in all particulars our earlier preparation. The organic matter contained 1.55 per cent N. It had still 2.19 per cent ash. Its optimum activity at 24° C. was $K = 0.0435$. It still gave the biuret with very great faintness. The other preparation IV A (3) was a similar white powder containing 2 per cent ash. The organic matter had 2.21 per cent nitrogen and its activity at 24° was $K = 0.057$. This preparation was the most active we have had.

We have, then, reduced the nitrogen content and increased the activity at the same time. Preparation IV A (3) contains one-third as much nitrogen as O'Sullivan and Tompson's powder "B," obtained in a manner similar to ours, of which the activity is not quantitatively given. It contained six-tenths as much nitrogen as their preparations E and G, 45 per cent of their D and F. Our preparation at 15.5° should have an activity of about $K = 0.03$, whereas O'Sullivan and Tompson's K was about 0.09 at that temperature. Any further reduction below 2.2 per cent has resulted in lowering the activity.

(*a*) N = 2.2 per cent; $K_{24^\circ} = 0.057$: (*b*) N = 1.55 per cent; $K_{24^\circ} = 0.043$; (c) N = 3.69 per cent; $K_{15.5^\circ} = 0.09$ (O'Sullivan and Tompson): *d*-N = 4.94 per cent: $K_{15.5^\circ} = 0.089$ (O'Sullivan and Tompson).

If the preparation containing 1.55 nitrogen was again precipitated by alcohol to which a little sulphuric acid had been added to make it flock various precipitates were obtained ranging from 0.3 per cent to 1 per cent nitrogen and all of them very inactive. The smaller the nitrogen content became, the smaller was the activity, so that when the gum was reached with only 0.3 per cent nitrogen it was almost completely inactive. On the other hand gums containing still 1 per cent of nitrogen were very much less active than one would expect if the activity went strictly proportional to the nitrogen. It is necessary to assume if the active principle is the nitrogen-containing substance that in these cases not only did the gum lose its nitrogen-containing substance but the ferment present was less active than before.

By precipitation with acid alcohol a portion richer in nitrogen remains behind in solution while a substance poorer in nitrogen is precipitated.

(*b*) *The action of alcohol.*

Seven-tenths of a gram of the preparation IV B (1)containing 1.52 per cent nitrogen and an activity of 0.0435 at 24° was dissolved in 50 cc. of water, filtered from the very small flocculent residue of phosphates, and precipitated by 60 per cent alcohol to which a few drops of sulphuric acid were added to make the precipitate flock. The precipitate weighed when dried at 100°-105°, 0.4725 gram and contained 0.00427 gram nitrogen or about 0.9 per cent. The activity was very greatly reduced. In a second experiment 2 grams of the same powder, not filtered from the insoluble residue, were dissolved in 50 cc. of water and precipitated by the addition of 70 cc. of absolute alcohol. A white milky solution and a gummy precipitate were obtained. The milky fluid was poured off after 10 minutes and evaporated in a glass dish whereby it precipitated on the side of the dish. On evaporation to dryness the residue was brownish in color with a strong smell of peptone (pyrrol) or amino-acid residues. 0.7372 gram yielded 0.1296 gram N or 1.77 per cent. The alcohol precipitate weighed something over 0.8 gram. 0.7990 gram was taken for analysis after drying at 100° for four hours; 0.0907 gram N was obtained or 1.13 per cent. The precipitate had lost most of its activity.

This experiment shows that further purification by alcohol after the nitrogen has reached 2.2 per cent throws down a precipitate with about one-third to two-thirds the nitrogen content of the original and with very little inverting power. The portion left in the solution contained more nitrogen, but appeared to be not very active, although accurate determinations of its activity were not made. The alcohol apparently destroys the activity of the ferment.

We then tried to see if boiling would destroy the ferment and free the gum from its nitrogen at the same time. 0.7 of a gram of the preparation IV B 1 containing 1.55 per cent N was dissolved in 50 cc. H_2O and 1 cc. $\frac{N}{10}$ H_2SO_4 added and boiled two minutes (some residue undissolved). After two minutes 1 cc. of this solution was added to 5 cc. of sugar solution. A slow inversion took place, which seemed more active than the acid alone would cause although no quantitative test was made, showing the ferment was not entirely destroyed. The solution of boiled invertase was cooled and precipitated by 60 per cent alcohol, the gum washed with absolute alcohol, dried in vacuum, and then at 105° for two and a half hours. Of the precipitate 0.4381 gram yielded 0.00428 gram N or 0.98 per cent. The ferment has, therefore, lost its activity, and diminished in nitrogen, but not to a greater amount than by alcohol precipitation alone.

5. THE NATURE OF THE NITROGEN-CONTAINING SUBSTANCE.

Since O'Sullivan and Tompson ascribe to their albuminoid a formula of molecular weight about 190 and percentage composition corresponding closely to an amino-tyrosin, and since the ferment gave a weak tyrosin and xanthoproteic reaction, it was at first thought that all the nitrogen might be in the tyrosin or phenylalanine group. The attempt was then made to determine the amount of tyrosin in the molecule by measuring it colorimetrically by the Duboscq colorimeter. Two preparations were taken, one containing 1.55 per cent nitrogen and fairly active; the other contained 0.98 per cent nitrogen and was almost inactive. About 0.5 gram of the substance, dried for several weeks over calcium chloride and finally dried in the hot air oven at 103° for three hours, was dissolved in 25 cc. of 13 per cent nitric acid heated to boiling and the solution after cooling made slightly alkaline with strong ammonia, and made to exactly 100 cc. in each case. The solutions thus prepared were compared with standard solutions made in the same way from weighed amounts of pure tyrosin prepared from horn. The determinations were uniform and it was found possible to determine tyrosin (or phenylalanine) in this manner very easily and quickly and with a good deal of accuracy when a solution of known strength was compared with another, unknown, of about the same strength. In this way the 1.55 per cent N enzyme was found to contain 0.34 per cent of tyrosin or phenylalanine. The 0.98 per cent N enzyme contained only 0.20 per cent tyrosin. If the protein contains 16 per cent N and its amount is 6.25 × N grams, this would give in the one case 3.4 per cent tyrosin and 3.3 per cent in the other. The protein contains, therefore, at most about 3.5 per cent of aromatic radicles. Tyrosin and phenylalanine make, therefore, but a small part of its molecule.

6. FURTHER OBSERVATIONS ON THE COMPOSITION OF THE GUM-FERMENT COMPOUND.

It is clear that what we and others have considered as invertase is a compound, or mixture, of a gum, and a protein and possibly some other substances. We have endeavored to determine whether this is all there is in the ferment or whether there are also other components, some of which might be active.

Salkowski[1] determined the gum in such a mixture by precipitating with Fehling's solution and, after freeing from copper, determining the gum by direct weighing. The two determinations did not agree very well. By one he got 53.47 per cent of the invertase as gum; and in the other 65 per cent. This would leave for the remainder of his preparation in one case a substance with 10 per cent of nitrogen and in the other 16 per cent of nitrogen, since his original preparations contained 8 per cent and 6 per cent of nitrogen. This would give in one case 65 per cent of gum and 35 per cent of protein, but in the other case there is a considerable deficit not accounted for. Salkowski considered the gum to be present as an impurity and not in combination with the ferment.

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[1] Salkowski: *Zeitschrift für physiologische Chemie*, xxxi, p. 305, 1900.

We made several determinations of the sugar obtained from the ferment by inversion with hydrochloric and sulphuric acids. The sugar is mainly mannose, but probably also in part glucose. Since mannose and glucose have almost identical reducing powers we have used Munson and Walker's[1] tables for glucose in computing the sugar from the cuprous oxide. To get complete inversion it is necessary to boil for thirty hours in $\frac{N}{10}$ sulphuric acid.

A gum (XI, which contained 0.90 per cent of nitrogen by Kjeldahl, was dried two weeks *in vacuo* over sulphuric acid. 0.0530 gram was dissolved in 50 cc. of $\frac{N}{10}$ sulphuric acid and boiled under a reflux condenser gently for 36 hours. At the end of that time it was neutralized with strong sodium hydrate and treated with Fehling's solution according to Munson and Walker. The cuprous oxide weighed 0.0517 mg. which corresponds to 22.1 mg. of glucose. Since only half was taken for analysis this would give 44.2 mg. all told or 83.4 per cent of the weight of the gum. Since the gum contained 0.9 per cent of nitrogen, multiplying by 6.25 would give 5.62 per cent of protein. The gum contained 1.34 per cent of ash. If the gum has a molecular weight of about 1880 as O'Sullivan and Thompson thought, and we accordingly eliminate the value of 9 molecules of water, between each pair of glucose molecules, the glucose would account for only 76 per cent of the original gum molecule, leaving a deficit of about 17 per cent of the weight unaccounted for.

Another sample gave very similar results, *i.e.*, glucose 83 per cent; nitrogen, 0.92 per cent; ash 2 per cent. Another sample containing 1.40 per cent nitrogen yielded only 76 per cent of its weight as glucose. It is possible that some water was still present in the gums since it was found impossible to dry the gums to constant weight without prolonged heating and without some browning. We felt no certainty that the water thus lost was not being set free by a slow decomposition of the gum. We have, therefore, preferred to use the analyses made with preparations after standing *in vacuo* at room temperature over sulphuric acid or calcium chloride for several weeks. In any case it is clear that assuming the nitrogen to be present as a protein, the reducing sugar accounts for about 76 per cent only of the weight of the gum. Even with a gum containing about 0.3 per cent of nitrogen, we obtained only about 77 per cent of the weight of the gum as glucose. We have thus a deficit of between 10 and 15 per cent of the weight of the invertase unaccounted for. We thought possibly some organic acid might be formed by hydrolysis, but experiment proved otherwise. Little acid was produced by forty-eight hours heating with 25 cc. of $\frac{N}{10}$ sulphuric acid 1.0195 grams of invertase. At the end it took 26.05 cc. of $\frac{N}{10}$ sodium hydroxide to neutralize the acid to a a faint pink with phenol-phthalein. The slight increase in acidity would be accounted for by the slight amount of phosphoric acid present.

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[1] Munson and Walker: *Journal of the American Chemical Society*, xxviii, p. 663, 1906.

We thought alcohol used in precipitating might be either absorbed or combined. As a matter of fact a gum preparation dried for two months over calcium chloride *in vacuo*, gave, when hydrolyzed with $\frac{N}{10}$ sulphuric acid and distilled, a small amount of iodoform, but the amount was unimportant.

We are unable then, to account for from 10–15 per cent of the invertase in the recognizable products. A part of this may be included water, but we do not think all of it is. It is, however, noteworthy that the inactive almost nitrogen-free gum gives almost as large a deficit as the active nitrogen-richer invertases. It seems probable, therefore, either that the method of analysis by hydrolysis and computation of the reducing sugar as glucose is not satisfactory, or else that there are some constituents of the gum, possibly mannite or other alcohols, which do not reduce Fehling's solution. All conclusions regarding the nature of invertase are rendered uncertain until this deficit is accounted for.

All attempts to split off or separate the active principle from the gum were failures, although Wroblewski states that he obtained a small quantity free from carbohydrate. This preparation was protein in nature and very active.

We attempted by precipitation with acid alcohol in the presence of a great deal of glucose to free the active principle from the gum. This attempt was unsuccessful, the ferment can only be precipitated by very large quantities of alcohol under these co ditions and alcohol destroys its action possibly by uniting with It is true, however, that the glucose preserves it somewhat from t alcohol action, but the precipitates always contained some gu Acids appear to split it off from the gum, but destroy its activit Acetone used as a precipitant gave an active precipitate whi always contained the gum. The attempt to separate it electr lytically was also a failure. The gum is not digested by diatase.

GENERAL CONCLUSIONS.

The facts presented in this paper, showing that the purest pre arations of invertase still continue to give a faint biuret and stronger Millon and xanthoproteic reaction as long as the ferme remains active and that there is a rough parallelism betwe nitrogen content and activity after the gum is fairly pure, certain suggest that the essential part of the ferment is a protein, althoug in view of the fact that 10–15 per cent of the molecule remai unaccounted for either as mannose or protein and that the pr liminary purification reduces nitrogen and increases the activit this conclusion must be regarded as tentative. Our purest a most active preparation contained one-third as much nitrogen O'Sullivan and Thompson's powder "B," prepared in a simil manner. They state that this was very active, but do not gi quantitative data. Our preparation was one-third as active their ordinary preparations which were not precipitated a obtained as powders, but were kept in solution. The organ matter in their solutions contained 3.69–4.94 per cent of nitr gen. In our invertase, we had between one-half and two-thir the nitrogen, but only about one-third the activity of th preparations. They state, however, that invertase, when pr cipitated by alcohol and dried as we prepared ours, lost some its activity. Our next most active preparation contained 1. per cent nitrogen and was correspondingly less active. Furth more, further precipitation by alcohol yields products poor nitrogen and weaker, so that finally gums are obtained alm free from nitrogen and entirely inactive.

This parallelism between activity and nitrogen content, wh it is not complete, nevertheless indicates that the active princi is a protein, possibly of an albumose or peptone nature, as Wroblewski thinks.

This general conclusion is on the whole supported by the known facts concerning the closely similar ferment diastase. Most observers have found it impossible to throw down the active principle of invertase without a great deal of carbohydrate gum with it,[1] but in diastase both Osborne[2] and Wroblewski[3] got the active principle free from carbohydrate and found it to be of a protein nature although they are not agreed as to the nature of the protein. Diastase appears to exist ordinarily in combination with a pentose gum.

While other interpretations are not definitely excluded until the other 10–15 per cent of the gum-invertase is accounted for, we suggest the following interpretation as that which explains nearly if not all facts regarding these two ferments and harmonizes the apparently contradictory results of various investigators:

What we ordinarily call an enzyme such as invertase, diastase, pepsin, etc., is a combination of a colloid with an active principle. The active principle is the enzyme itself and should of course be called the enzyme, but it has happened that the substances isolated as enzymes have been generally the combination of this active principle with the inert substance, colloidal in nature.

The colloidal part of the molecule which is inert might with propriety be called the zymophore or ferment bearer, since in cells most of the enzymes are probably thus united or borne, but as this word has been used by Ehrlich to designate the active principle itself, we may call the colloid simply the carrier or bearer, and the active principle, the enzyme or kinase.

The nature of the carrier varies with the different enzymes, but so far as the evidence permits a conclusion it appears generally to be related chemically to the substance (substrat) upon which the enzyme acts. Thus for carbohydrate enzymes the carrier is almost certainly a carbohydrate colloid. In the case of invertase it is a mannosan gum; in the case of diastase it is an arabosan gum. With pepsin and trypsin we should expect the carrier to be a protein and all observers agree that these ferments are protein in character. In the case of lipase, the carrier would on the basis of this reasoning be expected to be an ester not easily split by lipase, possibly a cholesterin ester. The difference between the carrier and the substrat is that the carrier is generally colloidal and the combination of the enzyme or kinase and carrier is stable whereas the substrat-enzyme compound is unstable. The fact that the active principle of invertase unites with all sorts of carbohydrates or polyatomic alcohols indicates that substances of this class may easily act as its receptor, to use Ehrlich's word, in the cell. The carrier then is generally of the nature of the substrat and it is this similarity which has given rise to the opinion that enzymes are chemically related to the substrats. The carrier part of their molecules appear to be generally so related.

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[1] Wroblewski states that in invertase obtained from Merck he got a small amount of invertase precipitated by ammonium sulphate, carbohydrate-free, a protein and very active.

[2] Osborne, T. B.: *Journal of the American Chemical Society*, xvii, p. 587, 1895, *Ibid.*, xviii, p. 1, 1896. *Berichte d. deutschen chem. Gesellschaft*, xxxi, p. 254, 1896.

[3] Wroblewski: *Berichte d. d. chem. Gesellschaft*, xxxi, p. 1128, 1898.

The nature of the active principle is less certain, but in the case of invertase and diastase it is probably a protein. Whether it is an albumose or peptone or coagulable albumin can not be said, although its resistance to alcohol precipitation, would appear to relate it to the peptones.

The combination of carrier and enzyme may be assumed to dissociate ordinarily very little; the enzyme carrier compound is stable and, as long as the enzyme is combined, inert. The dissociation is, however, greatly increased by acids in the case of invertase and by alkalies in some other cases, and the enzyme thus set partly or entirely free from the carrier, becomes capable of uniting with the appropriate substrat and bringing about its decomposition. We thus have an explanation of at least a part of the action of acids and alkalies in hastening or retarding the enzymatic reactions. The enzyme-carrier compound is probably what is ordinarily called a zymogen. In the free condition, however, the enzyme, which would probably then be extremely reactive, would be also very much less stable and easily decomposed by heat or light and easily enter into permanent stable union with other substances, such as alcohols, which would thus destroy its activity.

In the cell the various enzymes thus combined with the colloidal carriers of the cell and thus built into its structure may be moved about or accumulated in particular regions in an inert form, from which slight local changes in acidity set them free to produce their characteristic action at that particular point.[1]

This picture agrees in all essential points with Wroblewski's[2] interpretation of the way enzymes are locked up in cells[3] and called into activity by slight changes of acidity, only it clears up at once the seeming inconsistencies of the work of O'Sullivan and Tompson, Salkowski, Wroblewski, Osborne, and other observers, some of whom have regarded the enzyme as protein, others as carbohydrate and others as a union of a protein and carbohydrate.

That the foregoing conception closely approximates Ehrlich's general view is perfectly evident. In this ferment the mannosan gum or carrier is the cell receptor, the enzyme becomes the active side chain or zymophore or toxophore group. It is clear that any carbohydrate group might act as a receptor for this inverting enzyme, only if it happens to be mannosan, with which the ferment comes in contact it enters into a firm compound, inert toward others. If, on the other hand, the receptor is cane sugar, it will be at once broken up by the active principle.

O'Sullivan and Tompson believed that only when enzyme and carbohydrate carrier were united were they active. Osborne has made a similar suggestion that active diastase is a union of an albumose and a coagulable protein. Salkowski, on the other hand, thought the gum in invertase was only present as an impurity and not united with the ferment, and Wroblewski also thought that this was the case for invertase and that the pentose gum in diastase was only an impurity. There are several facts which seem to us to count strongly against the view that the gum is present entirely as an impurity, although we admit that some of the gum may be present uncombined with ferment and it is possible to split the gum from the ferment.

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[1] Nucleic acid may from this point of view be simply the carrier of some important ferment. Possibly protamine or histone is the enzyme. There are facts which would be easy to interpret on the basis that the cell chromosomes are such inactive compounds, the nucleic acid being the carrier and various active principles uniting with it.

[2] Wroblewski: *Journal für praktische Chemie*, lxiv, p. 1, 1901.

[3] Vernon: *Ergebnisse der Physiologie*, ix, 1910, "Intrazelluläre Enzyme," p. 176, has made a similar suggestion for proteolytic endoenzymes.

That the gum is probably both in diastase and invertase present as a compound with the enzyme is strongly indicated by the fact that both these enzymes are particularly noteworthy for their power of combining with a great variety of carbohydrates. It would be strange if just toward the carbohydrate ordinarily accompanying it, the ferment should show an inertness and lack of combining power. Another strong reason for supposing that the ferment and the gum are in combination is the fact that these gums are peculiar and are found associated with the ferment even in widely different cells. For example, the mannose gum is found in invertase from beets as well as invertase from yeast. Whether it occurs also in the invertase of the mammalian intestine is less certain, but it must be remembered that a gum of some sort can be isolated from the intestinal mucosa. We find a pentose gum associated with diastase from malt and diastase from *Aspergillus*, a mold. Whether pancreatic diastase contains a pentose gum is not certain, but it is certain that from the pancreas where diastase is, a considerable quantity of pentose is formed by hydrolysis. It is supposed at present that this comes from the nuclein of the pancreas, but it may also come from the diastase present. A pentose is also found in the liver, where again a diastase exists. Whether saliva contains a pentose is still uncertain. It seems to us improbable that the ferments from such widely different animal and plant cells should always contain these same gums, were they only present as impurities. Indeed this fact led us at first to suppose that the ferment must be the gum. The accelerating action of acids and the contradictory results of Wroblewski, Fraenkel and Hamburg and Osborne on diastase, are easier to understand on the hypothesis that the ferment is more active when split off from the gum or other carrier than when united with it, and that acids set it free.

Further work on this subject is in progress and other possibilities must be excluded before the view of the enzyme and carrier forming the zymogen, sketched above, can be definitely accepted, however attractive it may appear.

There still remains the possibility that the ferment action is independent of the nitrogen-containing group, but ordinarily associated with it. We are at present making the endeavor to duplicate Wroblewski's work on diastase and invertase and get the nitrogen-containing principle free from the gum and still active.

Could this be done it would go far toward settling the matter. Everything indicates, however, that the free ferment here as in the case of pepsin is unstable and its isolation will be a difficult matter.

SUMMARY.

1. Invertase has been prepared from yeast by O'Sullivan and Tompson's method of self-digestion and precipitation with alcohol. The invertase so prepared contains when most active about 1 per cent of ash, mainly phosphates, 2.2 per cent of nitrogen, and had an activity of $K = 0.057$ at 24° and in optimum acidity, where

$$K = \frac{1}{t \times C_{(\text{invertase})}} \log._{(10)} \frac{A}{A - x}$$

2. This invertase was one-half to two-thirds as active as O'Sullivan and Tompson's and contained one-third as much nitrogen as theirs. Other preparations contained 1.5–1.6 per cent nitrogen. These had an activity of about $K = 0.043$ at 24°. They contained one-fourth the nitrogen and were correspondingly less active.

3. Preparations containing less then 2.2 per cent of nitrogen were always less active. When less than 1 per cent of nitrogen was present they were almost inactive.

4. This invertase consists of a gum, a mannosan, and a nitrogen-containing portion. Seventy to 76 per cent by weight of the invertase was obtained as a reducing sugar (mannose in large part).

5. As long as they were active all preparations of invertase gave a very faint biuret, faint Millon and xanthoproteic reactions. The biuret is so weak that it can only be detected by careful observation. It is not given if the invertase is heated with sodium hydrate and copper sulphate. The test must be made at room temperature.

6. The invertase contained about 0.38 per cent of tyrosin or phenylalanine in a preparation containing 1.55 per cent nitrogen. Assuming the nitrogen to be present in a protein this would contain about 3.5 per cent tyrosin.

7. In view of these facts, invertase as ordinarily prepared is to be considered to be probably a union of a protein with a carbohydrate gum. The gum is a mannosan.

8. The rough parallelism between activity and nitrogen co tent, when the invertase is fairly pure, indicates that the acti principle is a protein.

9. All attempts to get the protein free from the gum and acti were failures. Acid alcohol destroys the activity.

10. In view of these facts and the observations of Wroblewsk Osborne and Fraenkel and Hamburg on diastase, it is suggested th what is ordinarily called invertase is a union of an inactive co loidal gum, with an active protein ferment. The active princip may be an albumose or a coagulable albumin. This union is ine and the ferment is thus tied up in the cell. The union of carrier an enzyme constitutes the invertase zymogen. By the action of aci the ferment is freed from its carrier, the gum, and becomes capab of uniting with and changing its substrat. The action of acids i cell-physiology, and in hastening the action of invertase and oth enzymes is thus partially explained. Diastase would appear to l a union of an albumose enzyme with a pentose gum. It is suggest that possibly the ferments are thus anchored and rendered ine in cells by uniting them with colloids. The name "carrier" suggested to cover these colloidal substances, the carriers of tl ferments. The carriers appear to be usually of the same chemic nature as the substrat of each ferment and to be colloids.

11. A little over 12 per cent of the weight of the invertase still unaccounted for, though a part of this may be water. Un this is accounted for, and until the parallelism between nitrog content and activity be found to be more exact the foregoi conclusion that the ferment is a protein must be tentativ Further work on the subject is in progress.

A METHOD FOR THE ESTIMATION OF REDUCING SUGARS.

By STANLEY R. BENEDICT.

(From Experiments Conducted in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York, under the Auspices of the George Crocker Special Research Fund).

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In previous communications[1] it has been pointed out that alkaline copper solutions in which carbonate is substituted for hydroxide are more delicate and specific as reagents for sugar detection than when alkalinity is secured by either sodium or potassium hydroxide, as in Fehling's solution. The carbonate gives ample alkalinity for the sugars to exhibit their full reducing power, but does not tend to decompose these substances, prior to their oxidation, to nearly so great an extent as does hydroxide.

The writer has previously proposed solutions for the detection and estimation of sugars, based upon the above stated observations.[1] The first solutions suggested in this connection were delicate and specific for sugar work, but possessed the disadvantage common to so many of the copper reagents in that they were not permanent after mixing. Subsequently it was found and reported[2] that a solution containing copper sulphate, sodium carbonate, and sodium citrate is more sensitive as a test for sugars than is Fehling's fluid, and unlike this latter solution, is not reduced by uric acid, chloroform, or the simple aldehydes, Furthermore, the citrate-carbonate-copper solution may be kept ready mixed without undergoing deterioration of any kind.

The present paper describes a modification of this solution which may be employed for the estimation of sugars. Like the writer's first solution for this purpose, this reagent is well suited to the ti tration of sugars because, upon its reduction, a white precipitat of cuprous sulphocyanate is produced, which permits the end-poin of the reduction to be readily observed. The formula and tech nique for the use of this solution are given below.

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[1] Benedict: This *Journal*, iii, p. 101, 1907.

[2] Benedict: *Ibid.*, v, p. 485, 1909.

The solution has the following composition per liter:

	grams
Copper sulphate (cryst.)	18.0
Sodium carbonate (cryst.)[1]	200
Sodium citrate	200
Potassium sulphocyanate	125
	cc.
Five per cent potassium ferrocyanide solution	5
Distilled water to provide a total volume of	1000

With the aid of heat dissolve the citrate, carbonate, and sul phocyanate in enough water to make about 800 cc. of the mixture and filter. Dissolve the copper sulphate separately in about 10 cc. of water and pour the solution slowly into the other liquid with constant stirring. Add the ferrocyanide solution cool, an dilute to exactly one litre. *Of the various constituents, only tl copper salt need be weighed with exactness.* Twenty-five cc. of th reagent are reduced by 0.050 gram of glucose, or by 0.053 grai of levulose.

Sugar estimations are conducted with the solution in the fo lowing manner: Measure 25 cc. of the reagent into a porcela evaporation dish (25–30 cm. in diameter) and add 10–20 grams crystallized sodium carbonate (or one-half the weight of tl *anhydrous* salt) and a *very small quantity* of powdered pumi stone. Heat the mixture to vigorous boiling over a free flame an run in the sugar solution quite rapidly until a heavy white preci itate is produced, and the blue color of the solution begins diminish perceptibly. From this point the sugar solution is ru in more and more slowly, with constant vigorous boiling, until tl disappearance of the last trace of blue color, which marks tl end-point.[2]

The following explanatory points may be added regarding tl solution. When ready mixed, like the qualitative reagent, th solution appears to keep indefinitely, without any special precautions, such as exclusion of light, etc. The trace of ferrocyanide is added to prevent precipitation of red cuprous oxide. which may be caused by certain impurities. *Chloroform has such a marked tendency in this respect that it must not be present during the titration.* The additional carbonate is added prior to the titration in order to provide sufficient alkalinity to insure the production of a sharp end-point.

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[1] One-half the weight of the *anhydrous* salt may be used.

[2] In applying this process to urine, the latter should be diluted, 1:1 unless the sugar content is known to be very slight.

As regards the accuracy of the process it may be noted that repeated determinations by different workers during the past year have convinced the writer that the method is probably more satisfactory than any other titration method for sugars at present available. Check determinations with the gravimetric (Allihn's) and polariscope processes have shown that the method gives highly satisfactory results, the figures in the various applications of it differing even less than those reported for the writer's previous quantitative sugar process. In this method, as in any other where the disappearance of color is made the end point, there is a tendency to run in an excess of the reacting substance unless special care is exercised throughout the titration and particularly at the end. The solution must be kept vigorously boiling over a free flame during the entire process, and towards the end the sugar solution must be added in portions of a drop or two, with an interval of about 30 seconds after each addition, Should the mixture become too concentrated during the titration process, distilled water may be added to replace the volume lost by evaporation.

REPLY TO THE PAPER OF BENSON AND WELLS, "THE STUDY OF AUTOLYSIS BY PHYSICO-CHEMICAL METHODS, II."

BY DR. RICHARD CHIARI.

(*From the Pharmacological Institute of the University of Vienna.*)

(Received for publication, December 26, 1910.)

In their recent paper[1] entitled "The Study of Autolysis by Physico-Chemical Methods, II," Benson and Wells have discussed the influence of antiseptics upon autolysis. They found that chloroform in comparison with other antiseptics, was most effective in inhibiting autolysis. The results of their experiments, as they affirm in a footnote, are quite contrary to those of my work.[2] The contradiction is, however, merely apparent and arises from a misunderstanding which I wish to explain.

My autolysis experiments were made with pieces of rabbit liver weighing 2.5–3.5 grams, placed in a 1 per cent solution of sodium fluoride. As a measure of the extent of autolysis I determined the nitrogen, non-precipitable by tannic acid, in the suspension of ground liver which was prepared after the completion of the autolysis.

I attempted to determine the *normal course of autolysis* and, in agreement with the results of Claypon and Schryver, was able to show a *latent period* of five to six hours. The evidence of this latent period is to be found in the fact that the amount of nitrogen, non-precipitable by tannic acid at the end of six hours, was very little if any greater than that of the soluble nitrogen contained in the fresh liver.

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[1] This *Journal*, viii, p. 64, 1910.

[2] Beeinflussung der Autolyse durch die Narotica der Fettreihe, *Arch. f. exp. Path. u. Pharm.*, lx, p. 256.

Benson and Wells did not find this latent period by their method, and from this I conclude that the processes of decomposition measured by their method are different from those measured by nitrogen estimations. For this reason alone the experiments are not comparable.

The course of autolysis proceeds apparently *in two phases*. The first, the latent period, comprises the solution (Lockerung) of the protoplasm during which *no differences* are to be observed by nitrogen estimations. In this stage, possibly, lactic acid or sugar is formed. Only in the second phase does destruction of the proteins occur. As I shall emphasize later, my principal consideration was the *influence exerted upon the first phase by the transitory action of the narcotics*. I exposed the pieces of liver to the fumes of the narcotics for two to three hours and then placed them in 1 per cent sodium fluoride in the incubator at the same time with the controls, *i.e.*, with pieces which had not been subjected to the fumes of the narcotics.

After 5 or 6 hours the pieces which had previously been subjected in this way to the action of the narcotics yielded much larger amounts of nitrogen not precipitable by tannic acid than did those not so treated, from which I conclude *that the latent period had been shortened, the process of autolysis accelerated by the previous action of these agencies.*

As controls, the amounts of nitrogen in the untreated pieces were given. The charge that control analyses are lacking is therefore quite unfounded. Again I wish to point out that in my experiments the subject under consideration was the *temporary influence of the narcotic fumes*, that during the actual process of autolysis no chloroform was added to the sodium fluoride solution. In this respect, also, my experiments were differently designed from those of Benson and Wells and, therefore, the results are not to be compared. It is shown on page 262 of my paper that *temporary treatment* with chloroform vapor does not depress the accelerated process of autolysis in frozen pieces of liver, *i.e.*, it does not influence the process of ferment action itself (second phase of autolysis).

Furthermore, this acceleration of autolysis is not without analogy in the action of lipoid solvents upon *living organisms*.[1] By short treatment with ether Johannsen was able to hasten the development of plants. By the short transitory action of these substances the cell contents are rendered more labile, metabolic changes and ferment activities promoted; the vital processes accelerated (der Inhalt der Zellen wird lockerer, Stoffumsätze, Ferment wirkungen werden ausgelöst und der latente Lebensprocess geweckt). Just as the vital process in plants is injured or brought to a standstill by *prolonged action of ether*, so autolysis, the ferment action itself, is inhibited by means of chloroform actually present, as Benson and Wells have shown in their paper.

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[1] Hans H. Meyer: *Münch. med. Wochenschr.*, 1909, p. 1577.

On the other hand, the short temporary action of narcotic vapors effects a solution of cell lipoids, *i.e.*, an *alteration* of the protoplasm itself, permitting the enzymes to have access to the protoplasm. In the experiments of Benson and Wells, they were dealing with *direct effects upon the enzymes;* in mine, however, the question is one of an *alteration in the state of the cells.*

ON NUCLEASES.

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It has been recorded by many observers that during the process of autolysis nucleins suffer a complete disintegration with liberation of phosphoric acid and of free purin and pyrimidin bases. The earliest records of such observations belong to Schutzenberg, to the pupils of Kossel and more particularly to Salkowski and his pupils. In more recent years and in greater detail the influence of autolysis on the nuclein-derivatives was studied by Kutscher, Dakin, Jones and Schittenhelm, and Levene.[1] This work, however, did not deal with individual factors concerned in the complete dissolution of the nucleic acid molecule, nor with the character of the intermediate steps leading to the final disintegration.

Attempts to obtain light on these phases of the problem were made only in recent years. The attempts to disclose the mechanism of the final phases in this complex process were crowned with success. Jones and his co-workers in this country and Schittenhelm with co-workers in Germany have succeeded in bringing much light into the mechanism of purin metamorphosis through the enzymes of animal tissues. The knowledge of the phases leading to the liberation of the purin bases remains less satisfactory, notwithstanding the very meritorious work of Araki, F. Sachs, Nokahama, Corbone, Iwanoff and others.

The failure of these investigations to furnish more satisfactory information on the details of the nucleic acid dissolution may be attributed to two factors, namely to the absence of a convenient method for the study of the changes in comparatively short in vals of time, and second to the lack of knowledge of the chemi structure of nucleic acid. In fact the results obtained by Sachs h to be appreciated all the more if the methods that were availa to him are taken into consideration. After the present work already completed and reported at a meeting at New Hav there appeared a publication by Giacomo Pighini,[2] who appl the optical method to the study of the nuclease action. It m be remarked, however, from the work of Pighini that he failed appreciate the fact that the results of his observations can be in preted correctly only if the rotatory power of the possible cleav products are taken into consideration. The work of Abderhal and his co-workers on the enzymatic hydrolysis of optically act polypeptides emphasizes this point very strongly.

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[1] A review of the literature is given in the article on "Autolysis" by P. A. Levene, *Harvey Lectures*, i, p. 89, 1906, Lippincott, Philadelphia and London.

Through the work of Jacobs and of one of the present writ the knowledge of the constitution of some nucleic acids advanced to a degree which makes possible an intelligent inter tation of the observation made on the process of nucleic a dissolution. Namely it was demonstrated that the molecule of complex nucleic acids is composed of nucleotides and these of p phoric acid, carbohydrate and base linked one to another in order here given. It was further proven that by selecting methods of hydrolysis it is possible to detach from the comp either phosphoric acid alone, giving rise to a nucleoside, or a c plex of carbohydrate and base, or under other conditions i possible also to remove only the purin base thus forming a p phoric acid conjugated with a carbohydrate. The rotatory po of the nucleosides and of the d-ribose phosphoric acid—taken an instance—differ in direction and in magnitude, and the r tory power of each one differs from that of d-ribose.

Thus the optical changes during the dismemberment of a sin nucleic acid, as for instance of inosinic acid, depend on the char ter of the product formed. The rotation of the entire comple $[\alpha]_D^{20} = -18.5$.[3]

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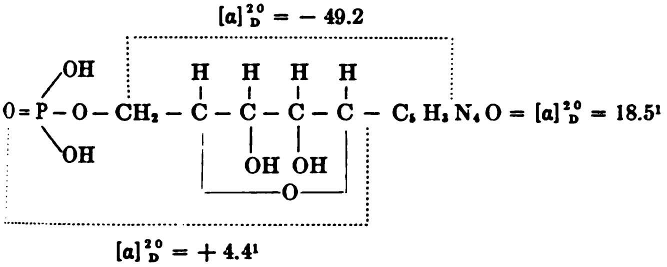
[1] We do not mention this with the intention of disputing priority.

[2] Giacomo Pighini, *Zeitschr. f. physiol. Chem.*, lxx, p. 85, 1910.

[3] For the barium salt.


$[\alpha]^{20}_{D} = -49.2$
OH
H H H H
$O=P-O-CH_2-C-C-C-C-C_5H_3N_4O = [\alpha]^{20}_{D} = 18.5$[1]
OH
OH OH
O
$[\alpha]^{20}_{D} = +4.4$[1]

That of d-ribose phosphoric acid = $[\alpha]^{20}_{D} = +4.4$[1] and that of inosin = $[\alpha]^{20}_{D}, = -49.2$ and that of d-ribose = $[\alpha]^{20}_{D} = -19.2$.

Thus, the transformation of inosinic acid into d-ribose phosphoric acid should lower the degree of the rotation of the solution, whereas the transformation into inosin should be accompanied by an increased rotation.

The interpretation of the results of observation on the yeast nucleic acid is much more complex for the reason that of the six products forming on its cleavage three: the d-ribose, guanosin, and adenosin, are levorotatory, while the other three, namely, d-ribose phosphoric acid, cytidin and uridin are dextrorotatory. From this it is obvious that an intelligent interpretation of the results obtained on the complex substances is possible only after the action of the same enzymes on the simpler substances has been established.

It was the object of the present investigation to observe the action of tissue extracts on the simpler complexes and then to apply the knowledge obtained in this manner for the interpretation of the observations to be made on the complex nucleic acids.

Substances employed in course of the experiments were the following: inosin, cytidin, inosinic acid, guanylic acid, yeast nucleic acid. Experiments with guanosin were abandoned for the reason of the great insolubility of the substance.

The action of the plasma of the following organs was tested: pancreas, liver, kidney, heart muscle, extract of the mucosa of the small intestines, and of blood serum. Only the organs of the dog were employed in the experiment.

The best *condition* for the action of the enzyme was found to be the neutral reaction of the medium as this is afforded by the phosphate mixture of Henderson and Webster.[1] The presence of acids or alkali either markedly lowered or completely arrested the action of the enzyme. On this point the results of the present experiments fully harmonize with those of Sachs. Only in one experiment the enzymotic action of the extract of the intestinal mucosa was so powerful that it was not arrested by the presence of acetic acid in $\frac{N}{80}$ (0.2 per cent) concentration. The dog whose organs were used in the experiment had been poisoned with cantharidin and had refused food for seven days.

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[1] The rotation was determined only for the barium salt.

The results of the experiments were all uniform and were the following:

Inosin. Plasma of heart muscle, of liver, kidney and of the intestinal mucosa invariably hydrolyzed it giving rise to the free base and d-ribose. The plasma of the pancreas, the blood serum and the hemolyzed blood remained without action on it.

Inosinic acid was hydrolyzed by the same organ plasmata as inosin, and as inosin remained intact after the action of the pancreas plasma, no experiments were performed with blood serum. The decline of the rotatory power observed during the experiment is gradual, constant, and the rotation always remained to the left. From this, one is justified to surmise that the products of hydrolysis are always phosphoric acid, d-ribose, hypoxanthin. There is no evidence of formation of inosin in any phase of the experiment, nor is there any satisfactory evidence for the assumption of formation of d-ribose phosphoric acid,—thus at all periods of the experiment the disruption of the molecule seems to be complete.

Guanylic acid. Great difficulties were encountered in the use of the acid for the reason of its strong tendency to gelatinize or to form precipitates with the enzyme solutions. However, one sample of the acid was obtained which furnished a satisfactory solution. The action of the plasma of the liver, kidney, heart muscle, and of the intestinal mucosa was very decisive, and of the same character as their action on inosinic acid.

The action of the pancreas plasma could be followed only once and in that experiment the change in rotation was not very high in value, but very decisive and showed an increase in levorotation.

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[1] Henderson and Webster: *Journ. of Med. Research*, xvi, 1907.

Should this observation be corroborated by further experience, it will justify the conclusion that guanosin is formed through the action of pancreas plasma on guanylic acid. Such a conclusion seems also to be in harmony with the observation of Levene and Jacobs[1] on the occurrence of free guanosin in the pancreatic gland. However, we realize that the observation needs further corroboration, and at present we are engaged in preparing guanylic acid suitable for the experiments.

Cytidin. From the as yet unpublished experiments of Jacobs, La Forge and one of the present writers it has become very probable that also cytidin is a complex of pentose and cytosin, though the two substances are united in a manner different from the glycosidic linkage. It is very significant therefore, that all the attempts to cause a cleavage of the substance by tissue plasma were futile.

Yeast nucleic acid. The action of the following substances was tested: the extract of intestinal mucosa, the plasma of the heart muscle, of the liver, of the kidney, of the pancreas, and blood serum.

The original solution of the nucleic acid, to which the enzyme had been added showed dextrorotation. In all experiments, with the exception of one, there was noted a marked fall in the dextrorotation. However, at the conclusion of the experiments the solution remained dextrorotatory. From this it follows that the cleavage of the nucleic acid under the conditions of the present experiments was not complete. Further, it is evident that substances with a lower dextrorotation than the original nucleic acid, or levorotatory substances are formed. It has become known from the experiments on the action of the extract of intestinal mucosa, of the plasma of the liver, kidney, and heart muscle on inosinic and guanylic acid, that these two simple nucleotides undergo complete disintegration; on the other hand, under the same influence cytidin remains intact. Thus it may be concluded that under the conditions of these experiments the yeast nucleic molecule decomposes into the following substances: phosphoric acid, purin bases, d-ribose, cytidin and uridin. A solution of these substances in proportion as they occur in the nucleic acid molecule is dextrorotatory.

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[1] Levene and Jacobs: *Biochem. Zeitschr.*, xxvii, p. 127, 1910.

A solution of the yeast nucleic acid suffers a decline in its dextrorotation also under the influence of pancreas plasma (in distinction from inosinic acid) and under the influence of blood serum. Under these conditions nucleosides do not undergo further cleavage, and therefore the cleavage of yeast nucleic acid under the same conditions cannot proceed beyond that phase. Whether or not it actually reaches that stage, as yet cannot be ascertained with certainty. If the observation on the action of pancreas plasma on guanylic acid shall be corroborated by further experiments it will lead to the conclusion that under the influence of pancreas extract and of blood serum, nucleosides are formed, which do not undergo further cleavage.

The presence of 2 per cent of sodium carbonate did not affect the enzyme action but such action was arrested by the presence of 2 per cent of acetic acid.

In the present experiments it was not possible to note a definite regularity in the velocity of reaction of the enzyme solutions. Experiments aiming to elucidate that phase of the problem are contemplated.

EXPERIMENTAL PART.

Preparation of organ-plasma. The procedure employed by Abderhalden in his work on proteolytic enzymes was followed very closely. Dogs of about 15–20 kilos in weight were used for the purpose. They were allowed to fast from one to two days, and bled to death under ether narcosis. The organs were removed observing as far as possible aseptic precautions. The adhering blood was removed by means of physiological salt solution, ground up with quartz sand and the plasma obtained by means of a Buchner press under a pressure of three hundred atmospheres. The solutions to which toluol and chloroform (1 per cent) were added, were allowed to autolyze at 37° C. for 18 to 20 hours, and filtered immediately before each experiment. Aseptic precautions were observed as far as the conditions of the experiments permitted.

Preparation of the extract of intestinal mucosa. The duodenum and the small intestines were used for this purpose. The organs were thoroughly washed with physiological salt solutions. The mucosa was separated, taken up in 100 cc. of 1 per cent phosphate solution, prepared according to Henderson, and allowed to stand under toluol for several hours. The solution was then filtered. When the solution was too strongly opalescent to allow polariscopic observations, it was diluted until the observations were made possible. Aseptic precautions were observed as far as conditions allowed.

Blood serum. The blood was received in sterile vessels, and the plasma separated from coagulum and from cells by centrifugalization.

Reaction of solution. Numerous experiments brought the conviction that the most favorable action was achieved at neutral reaction as it is obtained by the use of the Henderson phosphate solution. Sodium carbonate and acetic acid were employed when it was aimed to follow the influence on the enzymes of OH or H ions.

Observations were made in Landolt's polariscope. Special tubes 50 mm. long, with a capacity of 3 cc. were used. The tubes were provided with jackets filled with warm water. The solutions were kept at 37° C and under toluol, which was renewed from time to time. Unless turbidity made filtration necessary, the same tube without filtering its contents, or without disturbing it in any way, was used from beginning to the end of the experiment.

INOSIN EXPERIMENTS.

A. *In neutral phosphate solution (1 per cent).*

EXPERIMENTS WITH EXTRACT OF INTESTINAL MUCOSA.

Exp. I. XI, 17, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	1 hr.	3 hrs.	4½ hrs.	18 hrs.	42 hrs.	66 hrs.	90 hrs.
Exp.:	—0.95	−0.90	−0.74	−0.64	−0.10	cloudy	−0.08	−0.08
Control:	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Exp. II. XI, 26, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	9 hrs.	24 hrs.	31 hrs.	4
Exp.:	−0.96	−0.83	−0.81	−0.63	−0.35	−0.30	−
Control:	−0.02	−0.04	−0.04	−0.03	−0.06	−0.04	−

	72 hrs.	144 hrs.
Exp.:	−0.20	−0.16
Control:	−0.04	−0.04

Exp. III. XI, 11, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	hrs.	hrs.	6 hrs.	24 hrs.	36 hrs.	72
Exp.:	−0.94	−0.88	−0.65	cloudy	+0.12	+0.12	+
Control:	0.00	0.00	0.00	0.00	0.00	0.00	

	96 hrs.	120 hrs.
Exp.:	+0.10	+0.06
Control:	0.00	0.00

Experiments with Pancreas Plasma.

Exp. I. XI, 18, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.	72
Exp.:	−0.91	−0.91	−0.91	−0.91	−0.91	−0.93	−
Control:	+0.08	+0.08	+0.08	+0.08	+0.08	+0.05	+

Exp. II. XI, 26, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	9 hrs.	24 hrs.	31 hrs.	48
Exp.:	−0.92	−0.92	−0.92	−0.92	−0.90	−0.88	−
Control:	+0.02	+0.02	+0.02	+0.02	+0.02	+0.02	+

	72 hrs.	120 hrs.
Exp.:	−0.82	−0.83
Control:	+0.02	+0.02

Experiments with Liver Plasma.

Exp. I. XI, 18, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Inosin solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	20 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	72 hrs.
Exp.:	−0.70	−0.50	−0.50	cloudy	cloudy	−0.18
Control:	+1.18	+1.18	+1.18	+1.18	cloudy	+1.16

Exp. II. XI, 26, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Inosin solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	20 min.	2 hrs.	24 hrs.	31 hrs.	48 hrs.	72 hrs.	96 hrs.
Exp.:	−0.72	cloudy	cloudy	0.00	+0.02	0.00	0.00
Control:	−0.10	−0.10	cloudy	cloudy	−0.08	−0.08	−0.08

EXPERIMENTS WITH HEART MUSCLE PLASMA.

Exp. I. XI, 18, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.
Exp.:	−0.88	−0.75	−0.56	cloudy	cloudy	−0.10	−0.10
Control:	−0.03	−0.03	−0.02	−0.02	cloudy	−0.03	−0.03

Exp. II. XI, 26, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Inosin solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.
Exp.:	−0 75	−0 71	−0 71	cloudy	−0 56	−0.36	−0.36	−0.36
Control:	−0.04	−0.04	−0.04	−0.04	cloudy	−0.04	−0.04	−0.04

EXPERIMENTS WITH KIDNEY PLASMA.

Exp. I. XI, 18, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Inosin solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	72 hrs.
Exp.:	−0.70	−0.50	−0.40	cloudy	cloudy	−0.10
Control:	−0.06	−0.08	−0.08	−0.08	cloudy	−0.05

Exp. II. XI, 26, '10. Same concentrations as in Exp. I.

	30 min.	2 hrs.	4 hrs.	8 hrs.	23 hrs.	30 hrs.	47 hrs.
Exp.:	−0.84	−0.66	−0.60	cloudy	−0.10	−0.06	−0.02
Control:	−0.06	−0.06	−0.04	−0.04	−0.04	−0.04	−0.04

	71 hrs.	143 hrs.
Exp.:	−0.04	−0.02
Control:	−0.04	−0.04

EXPERIMENTS WITH BLOOD SERUM.

Exp. I. XII, 15, '10. Blood serum, 1 cc.
Inosin solution, 3cc.

Control: Blood serum, 1cc.
Phosphate solution, 3cc.

	10 min.	3 hrs.	6 hrs.	20 hrs.	26 hrs.	48 hrs.	72 hrs.	120 hrs.
Exp.:	−0.80	−0.78	−0.78	−0.78	−0.82	−0.82	cloudy	−0.83
Control:	−0.42	−0.44	−0.44	−0.45	−0.45	−0.45	−0.45	−0.45

Exp. II. XII, 29, '10. Same concentrations as in Exp. I.

	10 min.	5 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
Exp.:	−0.90	−0.92	−0.91	−0.92	−0.92	−0.92
Control:	−0.32	−0.33	−0.35	−0.35	−0.35	−0.35

EXPERIMENT WITH HEMOLYZED BLOOD CORPUSCLES.

(With distilled water and ether)

Exp. XII, 29, '10. Hemolyzed blood, 0.5 cc.
Phosphate solution, 0.5 cc.
Inosin solution, 5 per cent, 3.0 cc.

Control: Hemolyzed blood, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	1 hr.	3 hrs.	24 hrs.	40 hrs.	72 hrs.	144 hrs.
Exp.:	cloudy and dark	−0.90	−0.88	−0.88	−0.90	−0.88	−0.90
Control:	cloudy and dark	cloudy	cloudy	+0.06	+0.02	0.00	0.00

B. *In alkaline solution.*

Experiments with Extract of Intestinal Mucosa.

Exp. XII, 16, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc. in (1) 0.5 per cent sodium carbonate solution; in (2) 1 per cent sodium carbonate solution; in (3) 2 per cent sodium carbonate solution.

Control: Enzyme solution, 1 cc.
Inosin 5 per cent in phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	20 hrs.	28 hrs.	42 hrs.	68 hrs.	92 hrs.
Exp. (1):	−1.02	−1.02	−1.02	−0.86	−0.82	−0.80	−0.74	−0.70
Exp. (2):	−1.24	−1.24	−1.24	−1.19	−1.19	−1.19	−1.17	−1.12
Exp. (3):	−1.16	−1.16	−1.16	−1.13	−1.13	−1.11	−1.01	−0.98
Control:	−0.93	−0.85	−0.68	−0.40	−0.30	−0.20	−0.19	−0.19

Experiments with Heart Muscle Plasma.

Exp. XII, 16, '10. Enzyme solution, 0.5 cc.
Sodium carbonate solution, 0.5 cc.: in (1) 0.5 per cent; in (2) 1 per cent; in (3) 2 per cent.
Inosin solution, 5 per cent, 3 cc. in sodium carbonate solution: in (1) 0.5 per cent; in (2) 1 per cent; in (3) 2 per cent.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Inosin 5 per cent in phosphate solution, 3 cc.

	10 min.	2 hrs.	5 hrs.	20 hrs.	44 hrs.	70 hrs.	92 hrs.
Exp. (1):	−0.90	−0.95	−0.96	−0.94	−0.94	−0.94	−0.94
Exp. (2):	−1.16	−1.18	−1.18	−1.25	−1.25	−1.25	−1.25
Exp. (3):	−1.10	−1.12	−1.25	−1.25	−1.26	−1.26	−1.26
Control:	−0.86	cloudy	cloudy	cloudy	−0.72	−0.63	−0.38

C. *In acid solution.*

Experiments with Extract of Intestinal Mucosa.

Exp. I. XI, 11, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent in acetic acid, 0.2 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Acetic acid, 0.2 per cent, 3 cc.

	20 min.	2 hrs.	4 hrs.	6 hrs.	24 hrs.	48 hrs.	120 hrs.
Exp.:	−0.82	−0.67	−0.60	−0.45	−0.35	−0.22	−0.18
Control:	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Exp. II. XII, 16, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc. in acetic acid solution: in (1) 0.2 per cent: in (2) 0.4 per cent; in (3) 0.8 per cent.

Control: Enzyme solution, 1 cc.
Inosin 5 per cent in phosphate solution, 3 cc.

	10 min.	2 hrs.	5 hrs.	20 hrs.	28 hrs.	42 hrs.	68 hrs.	120 hrs.
Exp. (1):	−0.92	−0.92	−0.92	−0.92	−0.90	−0.90	−0.88	−0.88
Exp. (2):	−0.86	−0.86	−0.87	−0.85	−0.86	−0.86	−0.86	−0.86
Exp. (3):	−0.86	−0.86	−0.86	−0.89	−0.92	−0.92	−0.90	−0.92
Control:	−0.92	−0.85	−0.66	−0.40	−0.30	−0.20	−0.19	−0.19

EXPERIMENTS WITH HEART MUSCLE PLASMA.

Exp. XII, 16, '10. Enzyme solution, 0.5 cc.
Acetic acid solution, 0.5 cc.: in (1) 0.2 per cent; in (2) 0.4 per cent; in (3) 0.8 per cent.
Inosin solution, 5 per cent, 3.0 cc. in acetic acid solution: in (1) 0.2 per cent; in (2) 0.4 per cent; in (3) 0.8 per dent.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Inosin 5 per cent in phosphate solution, 3.0 cc.

	10 min.	2 hrs.	5 hrs.	20 hrs.	44 hrs.	70 hrs.	92 hrs.
Exp. (1):	cloudy	cloudy	−0.96	−0.97	−0.97	−0.98	−1.00
Exp. (2):	cloudy	cloudy	−0.92	−0.93	−0.93	−0.91	−0.92
Exp. (3):	cloudy	cloudy	−1.00	−1.00	−1.00	−1.00	−1.00
Control:	−0.86	cloudy	cloudy	cloudy	−0.72	−0.63	−0.38

INOSINIC ACID EXPERIMENTS.

In neutral phosphate solution (1 per cent).

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA.

Exp. XII, 10, '10. Enzyme solution, 1 cc.
Sodium inosinate solution, 6 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	9 hrs.	12 hrs.	26 hrs.	48 hrs.	120 hrs.
Exp.:	−0.70	−0.70	−0.61	−0.49	−0.34	−0.25	−0.19
Control:	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Experiment with Pancreas Plasma.

Exp. XII, 10, '10. Enzyme solution, 1 cc.
Sodium inosinate solution, 6 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	8 hrs.	12 hrs.	24 hrs.	31 hrs.	48 hrs.	120 hrs.
Exp.:	−0.71	−0.71	−0.71	−0.64	−0.60	−0.61	−0.62	−0.64
Control:	+0.02	+0.02	+0.02	+0.02	+0.02	+0.02	0.00	0.00

Experiment with Liver Plasma.

Exp. XII, 10, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Sodium inosinate, 6 per cent, 3 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	12 hrs.	27 hrs.	48 hrs.	96 hrs.
Exp.:	−0.06	−0.06	−0.58	−0.43	cloudy	cloudy	−0.06	−0.06
Control:	−0.07	−0.06	−0.06	−0.06	−0.06	cloudy	−0.06	−0.06

Experiment with Heart Muscle Plasma.

Exp. XII, 10, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Sodium inosinate solution, 6 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	2 hrs.	6 hrs.	8 hrs.	31 hrs.	48 hrs.	96 hrs.
Exp.:	−0.62	−0.62	−0.58	cloudy	−0.50	−0.45	−0.53
Control:	−0.04	−0.04	−0.04	−0.04	cloudy	−0.05	−0.05

Experiment with Kidney Plasma.

Exp. XII, 10, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Sodium inosinate solution, 6 per cent, 3 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	12 hrs.	24 hrs.	96 hrs.
Exp.:	−0.65	−60.5	−0.65	−0.57	−0.50	cloudy, dark	cloudy, dark
Control:	−0.06	−0.04	−0.04	−0.04	cloudy	−0.04	−0.04

CYTIDIN EXPERIMENTS.

Neutral phosphate solution (1 per cent).

EXPERIMENTS WITH EXTRACT OF INTESTINAL MUCOSA.

Exp. I. XI, 17, '10. Enzyme solution, 1 cc.
Cytidin sulphate solution, 10 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4½ hrs.	18 hrs.	26 hrs.	70 hrs.	94
Exp.:	+0.69	+0.67	+0.60	+0.55	cloudy	+0.68	+
Control:	0.00	0.00	0.00	0.00	0.00	0.00	

Exp. II. XI, 26, '10. Enzyme solution, 1 cc.
Cytidin solution, 10 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	9 hrs.	24 hrs.	48 hrs.	72 hrs.	14
Exp.:	+0.85	cloudy	+0.83	+0.84	+0.87	+0.89	+
Control:	−0.02	−0.04	−0.03	−0.06	−0.04	−0.04	−

EXPERIMENTS WITH PANCREAS PLASMA.

Exp. I. XI, 18, '10. Enzyme solution, 1 cc.
Cytidin sulphate solution, 10 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.	72
Exp.:	+0.68	+0.68	+0.68	+0.68	+0.64	+0.69	cl
Control:	+0.08	+0.08	+0.08	+0.08	+0.08	+0.05	+

Exp. II. XI, 26, '10. Enzyme solution, 1 cc.
Cytidin solution, 10 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	9 hrs.	24 hrs.	31 hrs.	48 hrs.	12
Exp.:	+0.98	+0.98	cloudy	+0.94	+0.96	+0.96	+
Control:	+0.02	+0.02	+0.02	+0.02	+0.02	+0.02	+

EXPERIMENTS WITH LIVER PLASMA.

Exp. XI, 18, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Cytidin sulphate solution, 10 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	2 hrs.	4 hrs.	24 hrs.	72 hrs.
Exp.:	+0.54	+0.52	+0.52	cloudy	+0.55
Control:	+1.18	+1.18	+1.18	cloudy	+1.18

EXPERIMENTS WITH HEART MUSCLE PLASMA.

Exp. I. XI, 18, '10. Enzyme solution, 1 cc.
Cytidin sulphate solution, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	6 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.
Exp.:	+0.70	+0.55	+0.50	cloudy	cloudy	+0.62	+0.62
Control:	−0.03	−0.03	−0.02	cloudy	−0.03	−0.03	−0.03

Exp. II. XI, 26, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Cytidin solution, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	2 hrs.	24 hrs.	31 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp.:	+0.85	cloudy	cloudy	+0.90	+0.90	+0.90	+0.90
Control:	−0.04	cloudy	cloudy	−0.04	−0.04	−0.04	−0.04

EXPERIMENT WITH KIDNEY PLASMA.

Exp. XI, 18, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Cytidin sulphate solution, 10 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	2 hrs.	8 hrs.	24 hrs.	72 hrs.	96 hrs.
Exp.:	+0.63	+0.58	+0.52	cloudy	+0.62	+0.62
Control:	−0.06	−0.08	−0.08	cloudy	−0.06	−0.06

GUANYLIC EXPERIMENTS.

Neutral phosphate solution (1 per cent).

EXPERIMENTS WITH EXTRACTS OF INTESTINAL MUCOSA.

Exp. I. XII, 10, '10. Enzyme solution, 1 cc.
Sodium guanylate solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	20 hrs.	48 hrs.	72 hrs.	120 hrs.
Exp.:	cloudy	−0.36	−0.36	−0.24	−0.24
Control:	0.00	0.00	0.00	0.00	0.00

Exp. II. XII, 16, '10. Enzyme solution, 1 cc.
Sodium guanylate, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	9 hrs.	24 hrs.	48 hrs.	120 hrs.
Exp.:	−0.36	−0.36	cloudy	cloudy	−0.21	−0.19
Control:	0.00	0.00	0.00	0.00	0.00	0.00

EXPERIMENT WITH PANCREAS PLASMA.

Exp. XII, 10, '10. Enzyme solution, 1 cc.
Sodium guanylate solution, 3 cc., 6 per cent.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	1 hr.	8 hrs.	24 hrs.	48 hrs.	96 hrs.
Exp.:	precipitate filtered	−0.35	−0.37	−0.37	−0.41	−0.41
Control:	+0.02	0.00	0.00	0.00	0.00	0.00

EXPERIMENT WITH LIVER PLASMA.

Exp. XII, 10, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Sodium guanylate, 6 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	20 min.	2 hrs.	8 hrs.	24 hrs.	48 hrs.	96 hrs.
Exp.:	precipitate filtered	−0.40	−0.39	cloudy	−0.26	−0.26	−0.26
Control:	−0.07	−0.07	−0.06	−0.06	cloudy	−0.06	−0.06

EXPERIMENT WITH KIDNEY PLASMA.

Exp. XII, 10, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Sodium guanylate solution, 6 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	1 hr.	3 hrs.	8 hrs.	24 hrs.	48 hrs.	96 hrs.
Exp.:	precipitate filtered	−0.42	−0.30	cloudy	cloudy	−0.30	−0.30
Control:	−0.06	−0.04	cloudy	−0.04	−0.04	−0.04	−0.04

EXPERIMENT WITH HEART MUSCLE PLASMA.

Exp. XII, 10, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Sodium guanylate solution, 6 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	1 hr.	4 hrs.	24 hrs.	48 hrs.	96 hrs.^
Exp.:	precipitate filtered	−0.38	−0.38	cloudy	−0.32	−0.3
Control:	−0.04	−0.04	−0.04	cloudy	−0.05	−0.05

YEAST NUCLEIC ACID EXPERIMENTS.

Neutral phosphate solution (1 per cent).

EXPERIMENTS WITH EXTRACT OF INTESTINAL MUCOSA.

Exp. I. XII, 28, '10. Enzyme solution, 2 cc.
Nucleic acid solution, 4 per cent, 3 cc.

Rotation:

10 min.	2 hrs.	5 hrs.	20 hrs.	44 hrs.
+0.92	+0.53	+0.50	+0.31	+0.31

Exp.: II. XII, 29, '10. Enzyme solution, 1 cc.
Nucleic acid solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	5 hrs.	24 hrs.	30 hrs.	42 hrs.	48 hrs.	90 hrs.	144 hrs.
Exp.:	cloudy	cloudy	+0.42	+0.48	+0.58	+0.50	+0.33	+0.33
Control:	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

EXPERIMENT WITH PANCREAS PLASMA.

Exp. XII, 22, '10. Enzyme solution, 1 cc.
Nucleic acid solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp.:	precipitate filtered	+0.96	+0.72	+0.60	+0.50
Control:	+0.04	+0.04	+0.04	+0.04	+0.04

EXPERIMENT WITH LIVER PLASMA.

Exp. XII, 29, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Nucleic acid solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	20 min.	1 hr.	6 hrs.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp:	precipitate filtered	+1.08	cloudy	+0.90	+0.80	+0.60	+0.47	+0.49
Control:	+0.37	+0.37	+0.37	cloudy	cloudy	+0.34	−0.34	+0.34

EXPERIMENT WITH HEART MUSCLE PLASMA.

Exp. XII, 29, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Nucleic acid solution, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	3 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp.:	+1.25	+0.80	+0.75	+0.90	+0.75	+0.70	+0.64
Control:	−0.06	−0.06	−0.06	cloudy	cloudy	−0.06	−0.06

EXPERIMENT WITH KIDNEY PLASMA.

Exp. XII, 29, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Nucleic acid solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	3 hrs.	6 hrs.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp.:	+1.26	+0.74	+0.60	+0.46	+0.35	+0.34	+0.33
Control:	−0.09	−0.09	−0.08	cloudy	cloudy	−0.08	−0.08

EXPERIMENTS WITH BLOOD SERUM.

Exp.: I. XII, 29, '10. Serum, 1 cc.
Nucleic acid solution 5 per cent, 3 cc.

Control: Serum, 1 cc.
Phosphate solution, 3 cc.

	10 min.	1 hr.	5 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	144 hrs.
Exp.:	+1.25	+1.23	+1.10	+0.96	+0.85	+0.85	+0.72	+0.72
Control:	−0.33	−0.35	−0.35	−0.35	−0.35	−.035	−0.35	−0.35

Exp.: II. XII, 31, '10. Serum, 1 cc.
Nucleic acid, 5 per cent, 3 cc.

Control: Serum, 1 cc.
Phosphate solution, 3 cc.

	10 min.	4 hrs.	24 hrs.	48 hrs.	120 hrs.
Exp.:	+1.23	+1.15	+0.97	+0.88	+0.85
Control:	−0.43	−0.44	−0.46	−0.46	−0.46

YEAST NUCLEIC ACID.

In alkaline solution.

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA.

Exp. I, 2, '11, Enzyme solution, 1 cc.
Nucleic acid solution, 5 per cent in
Sodium carbonate, 2 per cent solution, 3 cc.

Rotation:

10 min.	2 hrs.	8 hrs.	24 hrs.	48 hrs.	120 hrs.
+2.28	+1.90	+1.66	+1.36	+1.22	+1.16

In acid solution.

Exp. I, 2, '11. Enzyme solution, 1 cc.
Nucleic acid, 5 per cent in
Acetic acid solution, 1.2 per cent, 3cc.

Rotation:

10 min.	2 hrs.	24 hrs.	48 hrs.	120 hrs.
filtered	+0.13	+0.10	+0.11	+0.11

ON THE DETERMINATION OF ALKYLAMINES OBTAINED FROM URINE AFTER KJELDAHL DIGESTION.

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The action of Kjeldahl digestion on some of the nitrogen compounds occurring in urine[1] has been studied in order to facilitate the investigation of the alkylamines which are obtained by distillation of urine that has been digested with sulphuric acid. It has been shown that, when treated according to Kjeldahl, creatine, creatinine, methylurea, lecithin and peptone will yield ammonia and alkylamine, while urea and uric acid as well as the amino-acids will yield only ammonia. Creatinine, a normal constituent of urine, would account for certain amounts of mono- and dimethylamine, these being formed according to the temperature at which the digestion is carried out; but a comparison of the amount of organic base as obtained after Kjeldahl digestion of urine with the amount which could be derived from creatinine treated in the same way, will show that there is a remainder which is not accounted for. A part of this remainder consists of trimethylamine, which is present in every normal urine after Kjeldahl digestion. As a condensation of the methyl groups of creatinine resulting in the formation of trimethylamine was not observed when its organic base was determined, it must be concluded that urine contains substances which split off trimethylamine.

Such energetic treatment as Kjeldahl digestion is not at all necessary to set free trimethylamine, as distillation of urine with a small amount of caustic alkali, or even aëration with the same, will liberate it, though in smaller quantity. Furthermore, it h been found in urine that has stood for some time, preserved by chloroform solution of thymol, where bacterial action cannot l considered the cause of decomposition.

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[1] On Alkylamines as Products of the Kjeldahl Digestion, This *Journal*, viii, p. 41, 1910.

If only primary, secondary and tertiary amines with the lowe alkyl groups were derived from urine after Kjeldahl digestio the estimation of the organic base would offer no difficulty; b the possibility must be considered that there may also be prese amines with higher alkyl groups. A preliminary experiment w made in order to throw some light on the character of the alk groups likely to occur: 100 cc. of normal urine were digested wi 100 cc. of sulphuric acid and catalyser (one hour), distilled, a separated from the ammonia in a 2000 cc. flask. In one-half i contents the organic base was determined and found to be = 3.6 c $\frac{N}{10}$ nitrogen. This organic base was redistilled into 5 cc. of $\frac{N}{10}$ hydr chloric acid and evaporated in a platinum dish. A small resid resulted, which was weighed when it appeared to be dry, and w found to be 0.0300 gm. As the nitrogen equivalent of 3.6 c $\frac{N}{10}$ acid = 0.00504 gm. the corresponding amount, expressed terms of the lowest representatives of the aliphatic series would

$NH_2CH_3.HCl$ = 0.0243 gm.
$NH_2(CH_3)_2.HCl$ = 0.02934 gm.
$N(CH_3)_3.HCl$ = 0.0344 gm.
$NH_2C_4H_9.HCl$ = 0.03943 gm.

The residue diminished during several weighings to such an exte that it did not seem possible to arrive at a constant weight by cc tinued heating on the steam-bath without considerable loss substance. The odor of trimethylamine was strong, and t difference in the several weighings evidently was due to the vo tilization of this base.

Therefore the weight first obtained from the residue does r admit of any conclusion as to its composition, as the weight the alkylamine hydrochlorides may have been increased by nc evaporated hydrochloric acid, or decreased by volatilized amin while a third factor, the hygroscopicity of the residue, tends furtl to complicate the estimation of its true value.

Finding, then, that the constant weight could not readily obtained, the residue, found by the last weighing to be 0.0235 gr was immediately dissolved in acidulated water, and distilled, yielding 3.15 cc. of $\frac{N}{10}$ nitrogen = 0.00441 gm. N. This would correspond to:

$NH_2CH_3.HCl$ = 0.02126 gm.
$NH(CH_3)_2.HCl$ = 0.0257 gm.
$N(CH_3)_3.HCl$ = 0.0301 gm.

Here the weight of the residue approaches the amount calculated for methylamine hydrochloride from the nitrogen found by distillation. This leads to the supposition that the organic base obtained from urine by Kjeldahl digestion and separation from the ammonia by mercuric oxide, consists mainly of alkylamines with low alkyl groups.

The following experiments were then made towards the investigation of the amount of organic base in several urines.

METHOD.

The determination of the organic base is carried out as follows: 5 cc. of urine are digested with sulphuric acid and copper sulphate till the liquid shows light yellow color. A digestion of about 25–30 minutes will be found to be sufficient. The total nitrogen then is distilled off into a 500 cc. graduated flask containing an excess of $\frac{N}{10}$ sulphuric acid. The amount of total nitrogen can now be determined by titration, but as several determinations on the same specimen are usually carried out, a separate total nitrogen determination is preferable.

The contents of the graduated flask are neutralized with a strong alkali. Five to ten cc. of the alkaline mixture, with water to the mark, and for each 10 cc. of the total nitrogen about 1.5–2 gm. of yellow oxide of mercury, are added. After being shaken for about one hour (the graduated flasks being wrapped in dark cloths and attached to a revolving disk) the mercuric oxide is allowed to settle. Twelve hours will be sufficient for this, though the finest particles of mercuric oxide will still float on top of the liquid. To retain these small particles and thus get a sharp separation of the liquid from the mercuric oxide, a dense filter is necessary, which may be prepared in the following way:

Into a two-hole stopper fitting the graduated flask, are inserted two tubes arranged as in a wash-bottle; the tube serving as an inlet for the air being short, the one for the outlet of the liquid a little longer and carrying, by means of a rubber stopper, a tube as large as the diameter of the graduated flask will permit, to receive the filter consisting of tightly packed absorbent cotton.

When air-blast is applied, the first part of the filtrate, liable to be slightly turbid and give the ammonia reaction with Nessler's reagent, is thrown away; but when 25–50 cc. have passed, the addition of Nessler's reagent will not cause any change of color.

Thus 250 cc. are collected, either in a graduated flask or by means of a pipette, and subjected to distillation. The organic base is received in 5 cc. of $\frac{N}{10}$ acid and titrated with $\frac{N}{10}$ alkali; the difference from 10 cc. will give the nitrogen value of organic base in 5 cc. of urine.

In some of the experiments, the digestions were carried out over the free flame, but to secure uniform conditions for several determinations on the same specimen, an electric stove (kryptol resistance) was used. The following table may show that this latter arrangement was satisfactory, as in two different sets of experiments for the same period of digestion, analogous results were obtained:

TABLE I.

Urine with large amount of organic base.

Total nitrogen in 5 cc. = 23.8 cc. $\frac{N}{10}$ N. Organic base in cc. $\frac{N}{10}$ N.

NO.	a			b		
	TIME	N	ORGANIC BASE	TIME	N	ORGANIC BASE
	min.			*min.*		
1				5	22.1	1.1
2	7	22.9	1.2			
3				10	23.4	1.2
4	17	23.65	1.2			
5				20	23.7	1.2
6	20	23.7	1.2			
7				25	23.8	1.2
8	30	23.8	1.3			
9	45	23.8	1.2			

TABLE II.

Normal Urine.

Total nitrogen in 5 cc. = 19.45 cc. $\frac{N}{10}$ N. Organic base in cc. $\frac{N}{10}$ N.

NO.	TIME	N	ORGANIC BASE
	min.		
1	20	19.45	0.65
2	25	19.45	0.60
3	30	19.4	0.65
4	35	19.45	0.6
5	40	19.45	0.6

The data in Table I are rather striking on account of the readiness with which the nitrogen and organic base are split off. The temperature after a five-minute digestion was observed and found to be 245°–250°. As urea decomposes at about 160° and this urine contained a considerable amount of methylurea, the presence of the latter must be made responsible for the rapid digestion.

In Table II the effect of different periods of digestion is demonstrated to show that a longer digestion at a relatively low temperature does not affect the amount of organic base.

In Tables III–VI, given below, the determinations of creatinine, ammonia, urea and uric acid were carried out according to Folin. The organic base is recorded as found in 5 cc. of urine. This amount, of course, includes the alkylamines derived from creatinine which theoretically would be one-third of its total nitrogen; but as demonstrated in a previous paper,[1] about one-sixth only of the total nitrogen of creatinine can be determined as organic base after a half-hour's digestion, therefore one-sixth of the creatinine nitrogen was subtracted from the amount of organic base, and this is stated in a special column.

In table III, the effect of the intake of methylurea is shown in the increased amount of alkalymine during the second and third days. Creatine, however, does not cause any variation in the amount of the alkylamine.

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[1] *Loc. cit.*

TABLE III.

All specimens made up to 2000 cc.

NO.	5 cc. TOTAL N	5 cc. ORGANIC BASE	TOTAL N	CREATININE	ORGANIC BASE N (Rest)	INTAKE
	cc. $\frac{N}{10}$ N	cc. $\frac{N}{10}$ N	gm.	gm.	gm.	
1	20.5	0.6	11.5	1.373	0.251	
2	21.8	1.5	12.2	1.409	0.753	5 gm. methy
3	19.7	0.8	11.05	1.620	0.347	
4	19.5	0.6	10.92	1.421	0.248	
5	19.5	0.6	10.92	1.446	0.247	2 gm. creati
6	22.6	0.6	12.65	1.604	0.237	

In Table IV, results are recorded obtained from a nitrogen diet, followed by a nitrogen-poor diet, terminated by a nitrc free diet. The output of organic base is fairly constant unti sixth day, when the intake of methylurea caused a sudden inc in the amount of alkylamine. In a lesser degree, this inc continues the seventh day, but the following day the val organic base approaches that of the first day of the experin though 2 grams of creatine were fed. This confirms the o vation made in the first experiment, that feeding creatine doe produce an increase of the organic base in urine.

TABLE IV.

NO.	DIET	AMOUNT	TOTAL N IN 5 cc.	ORGANIC BASE IN 5 cc.	TOTAL N	CREATININE	ORGANIC BASE N (Rest)	INTAKE
		cc.	cc. $\frac{N}{10}$ N	cc. $\frac{N}{10}$ N	gm.	gm.	gm.	
1	Meat (2½ lbs.)	2000	51.3	0.7	28.7	2.16	0.254	
2		2000	64.4	0.75	36.1	2.382	0.273	
3	Starch and little cream	2000	36.8	0.7	20.6	1.952	0.271	
4		2000	22.5	0.7	12.8	1.862	0.277	
5		2300	16.8	0.7	10.8	1.942	0.331	
6		2000	16.1	1.3	9.02	1.862	0.613	5 gm. me urea
7	Starch	2000	14.7	0.9	8.23	1.841	0.391	
8		2000	13.9	0.75	7.78	1.67	0.317	3 gm. cre
9		2000	11.0	0.75	6.16	1.67	0.317	

In Table V the effect of feeding methylamine hydrochloride is shown. The increase in organic base is exceedingly slight, though the alkylamine content of the methylamine hydrochloride was nearly equal to that of the methylurea fed in the previous experiments.

TABLE V.

All specimens made up to 2000 cc. Organic base separated from the ammonia in the urea determination in parenthesis.

NO.	TOTAL N IN 5 CC.	ORGANIC BASE IN 5 CC.	NH_3 IN 50 CC.	UREA IN 3 CC.	TOTAL N	CREATININE	ORGANIC BASE N (Rest)	INTAKE
	cc. $\frac{N}{10}$ N	cc. $\frac{N}{10}$ N	cc. $\frac{N}{10}$ N	cc. $\frac{N}{10}$ N	gm.	gm.	gm.	
1	22.0	0.5	11.5	10.5 (0.2)	12.32	1.80	0.169	2 gm. methylamine hydrochloride
2	22.85	0.65	11.6	9.8 (0.35)	12.8	1.688	0.260	
3	22.6	0.5	8.1	11.15 (0.35)	12.65	1.76	0.172	
4	21.0	0.5	8.3	11.0 (0.3)	11.76	1.706	0.175	

In Table VI the experiment of feeding methylurea was repeated and the usual separation of the nitrogen compounds was made, in order to find out whether the nitrogen obtained by the ammonia and urea determinations contained alkylamines. It was noticed

TABLE VI.

Organic base obtained by the ammonia and urea determinations in parenthesis.

NO.	AMOUNT	TOTAL N IN 5 CC.	ORGANIC BASE IN 5 CC.	NH_3 IN 50 CC.	UREA IN 5 CC.	URIC ACID	CREATININE	ORGANIC BASE N (Rest)	INTAKE
	cc.	cc. $\frac{N}{10}$ N	cc. $\frac{N}{10}$ N	cc. $\frac{N}{10}$ N	cc. $\frac{N}{10}$ N	gm.	gm.	gm.	
1	2000	23.8	1.3	7.5 (0.25)	20.0 (0.55)	0.66	1.906	0.610	5 gm. methylurea
2	2350	19.45	0.65	6.7 (0.2)	16.8 (0.25)	0.51	1.813	0.315	
3	2000	22.2	0.55	9.0 (0.2)	19.4 (0.25)	0.57	1.80	0.196	
4	2000	21.55	0.5	7.7 (0.25)	18.6 (0.2)	0.60	1.906	0.162	

that in the nitrogen obtained by aëration a small amount of org base was present, while in separating the nitrogen from the digestion larger quantities of organic base were obtained. indicates an apparently unavoidable imperfection in the met of the urea determination, as urea does not split off alkylam

Comparison of the results obtained from these experim leads to the conclusion that the amount of alkylamine obtai from urine by Kjeldahl digestion is fairly constant, though it be influenced by feeding certain nitrogen compounds contai alkyl groups.

ON THE RECOVERY OF ADENINE.

By GEORGE DeF. BARNETT and WALTER JONES.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

(Received for publication, January 9, 1911.)

Of the five physiologically important purine derivatives the one which can be most easily identified and accurately estimated is adenine. Both the free base and its salts with inorganic acids are easily soluble in hot water so that there is little danger of loss by retention in coagula or inclusion in animal charcoal,[1] two possible sources of error which must be carefully considered when dealing with xanthine, guanine, and uric acid. When submitted to the scheme of Krüger and Solomon,[2] whether in the presence or absence of other purine bases, adenine passes sharply into the hypoxanthine fraction, its conduct in this respect being in marked contrast to that of hypoxanthine[3] and guanine which divide themselves somewhat equally between the two fractions. In the subsequent phases of Krüger and Solomon's scheme adenine conducts itself equally well. It passes easily into solution with a slight excess of ammonia and remains dissolved after the free ammonia has been expelled. On the contrary, hypoxanthine requires for its solution much more ammonia than is commonly supposed and is partly precipitated when the ammonia is driven off. Finally, adenine may be precipitated from exceedingly dilute solutions (1:13,000) by the addition of sodium picrate. The picrate crystallizes from hot dilute picric acid solution in characteristic aggregates of very pale yellow silky needles which melt somewhat sharply near 280 degrees. This compound is so little soluble that mother liquors obtained in its crystallization give only a suggestion of a precipitate with silver nitrate and ammonia.[1]

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[1] By dissolving impure hypoxanthine in a slight excess of sulphuric acid and boiling with animal charcoal we have been able to prepare specimens of the base which give absolutely no reponse to the sensitive color and reaction for xanthine. Evidently the small amount of xanthine originally, present is taken out by the charcoal.

[2] Krüger and Solomon: *Zeitschr. f. physiol. Chem.*, xxvi, p. 373.

[3] While this is not in accord with Krüger and Solomon we have many times convinced ourselves of its truth. See also Bruhns: *Zeitschr. f. physiol. Chem.*, xiv, p. 535.

While possessing these analytical advantages, adenine continually accumulates in the form of its insoluble picrate. Even in the preparation of the base its picrate is unavoidable and to be of use must be converted into some soluble non-toxic salt. This is, usually accomplished by removing the picric acid with ether, but owing to the insolubility of adenine picrate, the ether is shaken with a solution of the salt in hot mineral acid. Aside from the annoyance of such a procedure, the great amount of ether which must be used causes an appreciable loss of adenine. These and other difficulties are avoided in the following method which serves for the convenient separation of adenine from picric acid without serious loss of the base.

A solution of adenine picrate in 10 per cent ammonia, so diluted that it contains about 0.5 per cent of adenine is treated with a solution of silver chloride in ammonia. A gelatinous precipitate is formed which is so highly colored as to suggest that adenine silver picrate has been precipitated; but an examination of the fluid will show that nearly all the picric acid has remained in solution.[2] The gelatinous precipitate is filtered off, suspended in boiling water and decomposed with hydrochloric acid. The pale yellow hot solution, which contains all of the adenine but only a small amount of picric acid, is filtered from the white silver chloride and allowed to cool, when a small amount of adenine picrate is deposited. This is filtered off and may be joined to a subsequent experiment or as a convenient excess of silver chloride is at hand, it may be immediately transformed. It would seem better to filter the adenine chloride solution after cooling and treat the residual mixture of adenine picrate and silver chloride directly with ammonia, but the removal of the picrate acid is more easily effected when the two substances are separately dissolved in ammonia and their solutions united.

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[1] Bruhns *loc cit.*, p. 533.

[2] Bruhns states that adenine silver picrate loses picric acid when treated with ammonia and there remains adenine silver colored yellow by inclusions of adenine silver picrate (*loc. cit.*, p. 557). The amount of importance which he attributes to this may be inferred from his use of the ordinary method (*ibid.*, p. 539).

Whatever alternative is adopted, the adenine chloride solution is shaken out with a small quantity of ether, neutralized with caustic soda and the adenine is precipitated with copper sulphate and sodium bisulphite. The copper compound is decomposed with sulphuretted hydrogen and the adenine which is obtained by evaporating the filtrate from silver sulphide is crystallized out of hot 5 per cent sulphuric acid.

The convenience of this procedure over the older method is apparent, and the following data will show that the loss of adenine is little more than that which is necessarily involved in the crystallization of the sulphate.

Initial adenine picrate	2.087	
Recovered from the sulphate mother liquors	0.379	
Sulphate equivalent to the difference	0.897	(90%)
Adenine sulphate after two recrystallizations	0.805	
Picric acid from ammoniacal filtrates	1.190	
Picric acid from the adenine solution	0.075	

In the analysis of animal tissues it does not usually happen that both hypoxanthine and adenine are encountered in the same experiment; so that if adenine has been precipitated as picrate hypoxanthine will not be found in the filtrate, while in the absence of adenine no picric acid is used and the search for hypoxanthine is not thereby complicated. But in an examination of muscle which as a rule is free from adenase, added adenine which survives the digestion must be precipitated with picric acid while the filtrate from this picrate contains the preformed hypoxanthine of the tissue together with the excess of picric acid which has been used. Again it commonly happens that in the use of Krüger and Solomon's scheme with tissue extracts some xanthine escapes into the hypoxanthine fraction and will finally be found in the filtrate from adenine picrate. Finally, where one is observing the slow action of adenase fluids are obtained which contain both picric acid and hypoxanthine.[1]

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[1] See Voegtlin and Jones: *Zeitschr. f. physiol. Chem.*, lxvi, p. 250.

In all such cases it is better not to remove the picric aci
the solution with ether but to precipitate the purine comp
with silver nitrate and ammonia. Here as in the case des
nearly all of the picric acid remains in solution and the
amount which is precipitated can be removed with a little
after the silver compounds have been decomposed with
chloric acid.

NOTE: Folin's method for the recovery of creatinine from cre
picrate by the use of potassium bicarbonate (*Zeitschr. f. physiol.*
xlvii, p. 235) cannot be applied to the case in question as the great
of the adenine remains with the potassium picrate.

ON THE COMBINED ACTION OF MUSCLE PLASMA AND PANCREAS EXTRACT ON GLUCOSE AND MALTOSE.

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(Received for publication, February 2, 1911.)

The discovery of pancreatic diabetes stimulated many investigations into the general problem of the influence of the pancreas on the process of sugar combustion on the animal organism. It seemed that the efforts of the investigators into this problem were rewarded by a very rapid and complete success. Several investigators almost simultaneously reached the conclusion that the pancreas contained a substance which facilitated the combustion of sugar by the muscle. However, to the brilliant work of O. Cohnheim[1] is due the principal credit for having advanced substantial evidence in support of the theory that the pancreatic gland contained a co-enzyme to the enzyme present in the muscle, and that by the combined action of these two, the reducing power of a sugar solution is caused to diminish. The views of Cohnheim were assailed by Claus and Embden[2] but gained general recognition through the ingeniousness of the experiments, planned and carried out by Hall.[3]

The results of the investigations, until that work, were convincing only in their contention that the combined action of pancreas extract and muscle plasma on a sugar solution leads to a fall in its reducing power. They contained no effort to elucidate the chemical process by which this phenomenon was accomplished. The present investigation was planned with a view to fill this gap in the knowledge furnished by the earlier experiments on the combined action of muscle plasma and pancreatic extract on a sugar solution, and to interpret by this method the process of sugar combustion in the animal organism. All attempts, however, were futile to detect carbonic, formic, acetic or lactic acids among the products resulting from the apparent disappearence of sugar under the conditions of two experiments of Cohnheim and of Hall. At first glance there remained two possible interpretations of the phenomenon of Cohnheim. One, that the reaction of oxidation did not proceed beyond the stage of formation of gluconic or saccharic acid, the other, that the disappearance of sugar was caused not by a degradation but by condensation of the glucose molecule. The records of the experiments of Hall contain data which lend more support to the second of the above hypotheses. The figures in the tables of Hall demonstrate that the highest percentage of sugar disappearance takes place when the mixture of muscle plasma and of pancreatic extract is allowed to act on a sugar solution of relatively high concentration.

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[1] Cohnheim, O.: *Zeitschr. f. physiol. Chem.*, xlii, p. 401, 1904, xlvii, p. 253, 1906.

[2] Claus and Embden: *Beitr. z. chem. Physiol. u. Path.*, vi, p. 214, 1905.

[3] Hall, G. W.: *Amer. Journ. of Physiol.*, xviii, p. 283, 1907.

The results of the present experiments have corroborated fully the observations of Hall on the influence of the sugar concentration on the rate of its disappearance and thus have lent support to the condensation hypothesis.

This view found further support in results of the following experiments:

1. The reducing power of a sugar solution, lowered by the combined action of muscle plasma and of pancreatic extract, was restored to its original height by boiling with return condenser for two hours in the presence of 1 per cent of hydrochloric acid.

2. The same end was achieved in the following manner. A concentrated sugar solution, which had lost part of its reducing power under the influence of muscle and pancreas mixture was diluted to ten times its original volume by means of a phosphate solution containing a new portion of the combined enzyme mixture. The mixture was then allowed to stand twenty-four hours.

3. It was possible to isolate from a sugar solution, which had lost part of its reducing power through the action of muscle plasma and pancreatic extract, an osazone, having the properties of a biosazone' slightly contaminated by glucosazone. The osazone isolated under these conditions contained 12.12 per cent of nitrogen, while glucosazone requires 15.6 per cent of nitrogen, and

biosazone 10.73 per cent of nitrogen. The method employed for isolating this osazone was practically the same that led Emil Fischer to the separation of isomaltose, and Craft Hill to the discovery of his disaccharide.

The observation that the combined mixture of muscle plasma and of pancreatic extract possessed the power to hydrolyze the disaccharide formed through its action on a concentrated solution of glucose, led the present writers to test the action of an identical mixture on a solution of maltose. It was noted that under these conditions maltose underwent hydrolysis. The magnitude increased with the dilution of the maltose solution. Under the action of pancreas extract alone, no change in the reducing power of the solution took place. Muscle plasma alone caused a rise in the reducing power of the solution, but, in a much less degree than did a mixture of muscle plasma and pancreatic extract.

On the other hand, each of the parts of the mixture alone, remained without effect on glucose.

EXPERIMENTAL PART.

Methods of Preparation of the Extract and of the Plasma.

a. Muscle Plasma. Rabbits were used in all experiments; they were bled to death. Skin and subcutaneous tissue was carefully removed and all muscle rapidly separated from bone and tendon. The muscle was passed through a hashing machine and weighed. An equal volume of 1 per cent phosphate solution (made up according to Henderson to contain 9 parts of disodium phosphate and 1 part of the monosodium salt) was added. If the solution remained acid more of a concentrated phosphate solution was added until the mixture reacted just alkaline to litmus. The mixture was then allowed to stand for about one hour at a temperature of 1° C., pressed through cheese cloth till residue remained fairly dry, then thoroughly mixed with sand and pressed by means of a Buchner press at a pressure of 300 atmospheres. All liquids were combined.

b. Pancreas Extract. Weighed pancreas of a rabbit was thoroughly ground up with sand, taken up in water, boiled and filtered. The residue was extracted repeatedly with alcohol.

The combined alcoholic and aqueous extracts were evaporated to dryness, taken up with water and filtered.

All operations were carried out with every possible aseptic precaution. Instruments, sand, measuring apparatus, etc., were sterilized. The parts of the Buchner press which came in contact with the muscle during the process of pressing were kept for twenty-four hours previous to the operation in 95 per cent alcohol, and washed with sterile phosphate solution immediately before the beginning of the experiment.

Toluol and chloroform were used as antiseptics. It was found that the addition of one or the other alone was insufficient to prevent bacterial growth.

The presence or absence of bacterial growth was ascertained by means of microscopical examination (smears) and by cultures. Only the solutions in which there was a complete absence of bacterial growth were taken into consideration.

The writers are greatly indebted to Drs. Lamar and Bronfenbrenner for the bacteriological examination of all solutions employed in this work.

SUGAR ESTIMATION. For the estimation of the reducing power of the solutions they were freed from protein by heat coagulation. A given quantity of the filtrate was boiled with Fehling's solution, the cuprous oxide filtered through a Gooch crucible; redissolved in nitric acid, reduced by sulphurous acid, and titrated according to the method of Volhard. Sufficient solution was taken in the comparative reduction estimations to make the difference in reducing power reach values, which would not be affected by possible errors of the method.

An attempt was also made to apply the optical method of measuring the sugar concentration. This method was not applicable to the present experiments for the reason that little change was observed in rotatory power of the sugar solutions even after a marked fall of its reducing power. This, however, is not surprising for the reason that the condensation product may possess a higher rotatory power than glucose.

Experiments showing the influence of the concentration on the rate of disappearance of glucose will not be reported in a separate paragraph, since evidence of it is contained in the experiments of all other series.

A. *Experiments in which an attempt was made to detect the products of oxidation of glucose.*

Twenty-five cc. of muscle plasma, 5 cc. of glucose solution containing approximately 5.0 gm. of glucose, 5 cc. of a 10 per cent phosphate solution, 5 cc. of pancreas extract (0.8 gm. of the gland to 100 cc. of muscle).

	GLUCOSE	GLUCOSE	DISAPPEARED	DISAPPEARED
	grams	*per cent*	*grams*	*per cent*
At the beginning of experiment	4.810	12.02		
12 hr. after beginning of experiment	4.120			
36 hr. after beginning of experiment	3.985	9.96	0.825	17.2

Thirty-five cc. of the solution which lost in reducing power a value corresponding to 0.5775 gm. of sugar was acidulated with phosphoric acid and distilled into $\frac{N}{10}$ barium hydrate solution. The distillate was filtered from barium carbonate by means of an arrangement which prevented, during the filtration, the access of the carbonic acid of the air. The filtrate was titrated back with $\frac{N}{10}$ hydrochloric acid, using phenolphtalein as indicator. The loss of barium hydrate caused by the carbonic acid of the distillate corresponded to 15.5 cc. of $\frac{N}{10}$ barium hydrate solution, or to 0.045 gm. of carbon dioxide. The control on the enzyme mixture without glucose was not made in this instance. The filtrate from the barium carbonate was again acidulated with phosphoric acid and distilled into $\frac{N}{10}$ sodium hydrate. No volatile acids distilled over.

The residue from the first steam distillation was extracted with ether according to the process of Buchner and Meisenheimer but no lactic acid was detected. To test the accuracy of the method it was applied to the extraction of lactic acid from Liebig's beef extract. The results were satisfactory.

Thus from this experiment it was evident that only a minimal quantity of carbonic acid could be obtained from the product of reaction of muscle plasma and pancreatic extract on a glucose solution. And regarding this the possibility was not excluded that the carbonic acid was present in the mixture at the beginning of the experiment.

The following experiment aimed to test this possibility. A mixture of muscle plasma and pancreatic extract in the same proportions as were used for acting on glucose solution was tested for the presence of glucose before and after hydrolysis with hydrocholoric acid. Notwithstanding the fact that 10.0 cc. of the solution were employed for each reduction experiment the result was negative.

A portion of the same mixture was allowed to act on a solution containing 19.0 per cent of glucose. The solution lost 11 per cent of its reducing power in 24 hours. Eighty cc. of this solution was acidulated with phosphoric acid and distilled with steam into $\frac{N}{10}$ barium hydrate solution. It required 18.5 cc. of the alkali to satisfy the carbonic acid distilled from the solution.

Ninety cc. of the original plasma and pancreatic extract solution distilled in the same manner developed an amount of carbonic acid equivalent to of 20.6 cc. of $\frac{N}{10}$ barium hydrate solution.

It followed from this experiment that the carbonic acid was not derived from of the disappearing glucose.

B. *Experiments aiming to establish the influence of hydrolysis by means of hydrochloric acid on a glucose solution acted upon by a mixture of muscle plasma and pancreatic extract.*

Experiment I. Sugar solutions of various concentrations were acted upon by the enzyme mixture. A sample of each mixture was analyzed for its sugar content at the beginning of the experiment, another after 24 hours and a third sample was taken at the same time, but previous to analysis it was hydrolyzed for two hours in the presence of 1 per cent of hydrochloric acid using a return condenser. Each solution, for analysis, was diluted in the following manner:

GLUCOSE CONCENTRATION APPROX.	VOLUME OF ORIGINAL SOL'N DESIRED FOR ANALYSIS	VOLUME OF ORIGINAL SOLUTION TAKEN	DILUTED TO	VOLUME OF DILUTED SOLUTION TAKEN FOR ANALYSIS
per cent	*cc.*	*cc.*	*cc.*	*cc.*
20	0.25	5	100	5
15	0.50	5	100	10
10	1.00	10	100	10
5	2.00	10	50	10
2	4.00	10	50	10

In the following tables are given the volume of each solution employed for the reduction test, the volume of sulphocyanide solution that it required to titrate the cuprous oxide reduced by it, the corresponding value calculated for one cc. and the calculated sugar concentration.

EXPERIMENT I.

	CC. EMPLOYED	CC. NH_4CNS	NH_4CNS PER CC.	PER CENT OF GLUCOSE	LOSS PER 100 CC.	PERCENTAGE LOSS
a. At beginning of experiment.....	0.25	20.5	82.0	28.7		
After 48 hours..................	0.25	20.5	82.0	28.7	0	0
b. At beginning of experiment.....	0.25	14.7	58.8	20.6		
After 48 hours..................	0.25	13.0	52.0	18.2	2.38	12.0
After hydrolysis................	0.25	14.6	58.4	20.3		
c. At beginning of experiment.....	0.5	14.5	29.0	10.15		
After 48 hours..................	0.5	13.1	26.2	9.1	1.0	11.0
After hydrolysis................	0.5	14.25	28.5	9.9		
d. At beginning of experiment.....	1.0	14.6	14.6	5.11		
After 48 hours..................	1.0	14.0	14.0	4.9	0.2	4.0

Thus in the last three dilutions it was noted that the highest proportion of disappearance of glucose corresponded to the highest concentration and the lowest with the greatest dilution. The absence of any action in experiment *a* lacks explanation.

EXPERIMENT II.

	CC. EMPLOYED	CC. NH_4CNS	NH_4CNS PER CC.	PER CENT OF GLUCOSE	LOSS PER 100 CC.	PERCENTAGE LOSS
a. At beginning of experiment......	0.5	34.0	68.0	23.8		
After 24 hours....................	0.5	31.7	63.4	22.19	1.61	7.3
After hydrolysis..................	0.5	33.7	67.4	23.59		
b At beginning of experiment......	0.25	12.1	48	16.8		
After 24 hours....................	0.25	11.1	44.4	15.3	1.5	9.4
After hydrolysis..................	0.25	12.0	48	16.8		
c At beginning of experiment......	0.5	15.9	31.4	10.99		
After 24 hours....................	0.5	15.5	31.0	10.35	0.64	6.0

EXPERIMENT III.

Glucose and Pancreas Extract.

	CC. EMPLOYED	CC. NH_4CNS	CC. NH_4CNS PER CC.	PER CENT OF GLUCOSE	LOSS PER 100 CC.	PERCENTAGE LOSS
At beginning of experiment.........	0.5	25.1	50.2	17.57		
After 48 hours......................	0.5	25.0	50.0	17.50	0	0

Glucose and Muscle Plasma.

At beginning of experiment.........	0.5	25.8	51.6	18.06		
After 48 hours......................	0.5	25.8	51.6	18.06	0	0

Glucose, Muscle Plasma and Pancreas Extract.*

At beginning of experiment.........	0.5	27.5	55.0	19.25		
After 48 hours	0.5	24.3	48.6	17.01	2.24	11.6

* 10 cc. of muscle plasma gave no appreciable reduction.

C. *Experiments aiming to show the action of muscle plasma and of pancreatic extract on the product of condensation of glucose.*

The first part of the experiment was performed in the same manner as in the experiment of the previous series. After the presence of condensation was demonstrated, the solution was diluted by means of one per cent phosphate solution, a new portion of plasma and of pancreatic extract was added, and the solution diluted to one-tenth of its original concentration. It was then allowed to stand in thermostat in the presence of toluol and chloroform. Before analysis the solution was again tested for the presence of bacterial growth.

EXPERIMENT I.

	CC. EMPLOYED	CC. NH_4CNS	PER CC.	PER CENT OF GLUCOSE	ABS. GAIN (+) OR LOSS (−)	PERCENTAGE GAIN OR LOSS
At the beginning of experiment......	0.5	28.0	56.0	19.60		
After 60 hours......................	0.5	24.0	48.0	16.80	−2.80	14.2
After hydrolysis....................	0.5	27.0	54.0	19.44	+2.64	94.2*

After dilution and continued action of the enzyme solutions.

At beginning of experiment..........	4.0	19.2	4.8	1.68		
After 36 hours......................	4.0	22.0	5.5	1.92	+0.24	14.1

*Calculated on basis of the total loss.

EXPERIMENT II.

	CC. USED	NH_4CNS USED	PER CC.	PER CENT OF GLUCOSE	ABS. GAIN (+) OR LOSS (−)	PERCENTAGE GAIN OR LOSS
At beginning of experiment..........	0.5	28.25	56.5	19.7		
After 144 hours.....................	0.5	25.6	51.2	17.9	−1.80	9.2
After hydrolysis....................	0.5	27.5	55.0	19.25	+1.35	75.0*

After dilution and continued action of the enzyme solution.

At beginning of experiment..........	5.0	25.6	51.2	1.79		
After 36 hours......................	5.0	28.2	56.4	1.97	+0.18	9.2

*Calculated on basis of the total loss.

D. *Separation of the biosazone formed from glucose by the c* *bined action of muscle plasma and of pancreatic extract.*

A solution of glucose which originally contained 12.0 gm.
glucose, was acted upon by muscle plasma and pancreas extr
It lost part of its reducing power corresponding to 10 per cent
glucose. The solution was made up to a volume of 120 cc. and
treated with 25.0 gm. of phenylhydrazine dissolved in glacial ac
acid. The solution was then placed in a boiling water bath. A
an hour the first precipitate of osazone was removed by filtrati
The filtrate placed on a water bath for another hour and
second precipitate of glucosazone was removed trom the
solution. This operation was repeated four times and the fi
hot filtrate was allowed to cool in the refrigerator at -1.0° C.
cooling an osazone separated out. This was removed by fil
tion, dissolved in alcohol, allowed to stand over night in the ref
erator. The clear solution was diluted with hot water, and on co
ing again an osazone crystallized. The treatment with alcohol
repeated. The final osazone was recrystallized from water c
taining pyridine. It consisted partly of microscopic plates. T
substance sintered at 190-195° C and had a melting point of 200°
(uncorrected.)

For analysis the substance was dried in a vacuum toluol b
over phosphorus pentoxide.

0.1000 gm. substance gave 10.7 cc. of nitrogen at 764 mm. and 22°

	Calculated for $C_{24}H_{32}O_9N_4$:	Calculated for $C_{18}H_{22}N_4O_4$:	Found:
N....................	10.73	15.56	12.12

In a second experiment performed in exactly the same man
the osazone contained 12.4 per cent of nitrogen.

E. *Experiments on the action of muscle plasma and of pancre* *extract on maltose.*

The experiments of this series were planned exactly in the sa
manner as those with glucose. Also the methods of analysis w
the same. In one experiment the action of the pancreas extr
alone and of the muscle plasma alone were tested. The resu
of the experiment are recorded in the following table.

Maltose and Pancreas Extract.

	cc. used	cc. NH$_4$CNS	cc. NH$_4$CNS per cc.	maltose gm. per 100 cc.	loss per 100 cc.	percentage loss
a. At beginning of experiment.......	2.5	18.8	7.52	4.36		
After 24 hours..................	2.5	18.7	7.48	4.33	0	0
b. At beginning of experiment.......	2.5	14.7	5.88	3.41		
After 24 hours..................	2.5	16.5	6.60	3.23	0.188	5.4
c. At beginning of experiment......	2.5	17.6	7.04	4.07		
After 24 hours..................	2.5	22.1	8.84	3.52	0.477	11.7

Maltose, Pancreas Extract and Muscle Plasma.

	cc. used	cc. NH$_4$CNS	NH$_4$CNS per cc.	maltose gm. per 100 cc.	loss per 100 cc.	percentage loss
a. At beginning of experiment......	0.5	18.7	37.4	21.69		
After 20 hours..................	0.5	18.7	37.4	21.69	0	0
b. At beginning of experiment......	1.0	25.5	25.5	14.79		
After 20 hours..................	1.0	26.0	26.0		0.120	0.81
c. At beginning of experiment......	2.0	26.6	13.3	7.71		
After 20 hours..................	2.0	27.8	13.9		0.152	1.9
d. At beginning of experiment......	4.0	23.4	5.85	3.39		
After 20 hours..................	4.0	26.6	6.65		0.210	6.2

GENERAL METABOLISM WITH SPECIAL REFERENCE TO MINERAL METABOLISM IN A PATIENT WITH ACROMEGALY COMPLICATED WITH GLYCOSURIA.

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In 1886 Marie had occasion to observe morphological changes in the pituitary gland in the course of acromegaly and on the basis of this, attributed to the organ an important function in regulating metabolism. The publication of his work stimulated much investigation into the rôle of the pituitary body in the metabolism in health and disease. The earliest of these researches belong to Schiff.[1] On two patients with acromegaly complicated with myxoedema there was noted a retention of phosphoric acid. On the same patient after treatment with tablets of the compressed pituitary gland, the phosphoric acid metabolism was altered, and a daily loss of the substance took place instead of the retention. A similar influence of the treatment with the gland was noted in a patient with paralysis agitans, while the metabolism of a normal individual was not affected by the treatment. The author interpreted these peculiarities of metabolism by the wasting of bones, for the reason that the change in the nitrogen output was insignificant as compared with that of phosphoric acid.

Four years later Moraczewski[2] tested the effect of treatment with the pituitary body on the metabolism of patient with acromegaly complicated with glycosuria. He noted a tendency towards retention of nitrogen and of chlorine and a very marked retention of phosphorus and of calcium. After treatment with tablets of the dried pituitary gland the nitrogen and chlorine output were reversed causing a negative balance of these substances, while in regard to phosphoric acid and to calcium there persisted a retention. In course of the experiment the patient lost 3 kilos in weight.

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[1] Schiff, A.: Hypophysis in ihrer Einwirkung auf den menschlichen Organismus. *Wien. klin. Woch.* No. 12, 1897.

Beeinflussung des Stoffwechsels durch Hypophysis- und Thyroidea Präparate. *Zeitschr. f. klin. Med.*, xxxii, Suppl. p. 289, 1897.

[2] v. Moraczewski: Stoffwechsel bei Akromegalie, *Zeitschr. f. klin. Med.*, xliii, p. 336, 1901.

Edsall and Miller[1] published their observations on two patients with acromegaly: one in the active, the other, in the resting stage. In both patients there was noted a retention of nitrogen, and of phosphoric acid. In the patient in progressive stage of the disease there was observed also a retention of calcium. The proportion of phosphoric acid retention was high as compared with that of calcium, which led the authors to the conclusion, that in course of the disease the hypertrophy was not limited to the bones, but extended to other tissues. The patients were maintained on a rich diet. Franchini[2] observed in one patient with acromegaly a retention of nitrogen, calcium and magnesium. The sulphur, chlorine and phosphorus metabolism was normal.

Mendel,[3] likewise observed a change in chlorine and phosphorus metabolism and a retention of nitrogen. Tauszk and Vas[4] reported a slight retention of nitrogen and of phosphoric acid, and an increase in calcium output. Treatment with tablets of pituitary body remained without influence on the general metabolism.

Recently very careful observations on a patient with acromegaly were made by Oberndorfer.[5] The results of his observations led him to no definite conclusion regarding the character of the metabolism in this condition. The variations in the nitrogen output were explained by the low intake. The slight retention of phosphoric acid and loss of calcium in the author's opinion did not exceed the variation in the output of these substances in health. The authors expressed a degree of scepticism also regarding the finding of definite abnormalities of metabolism in acromegaly by other writers. On the other hand a very recent private communication of Parhon corroborates the observation of those authors who report a retention of phosphoric acid and of calcium.

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[1] Edsall and Miller: Chemical Pathology of Acromegaly, *Med. Bull., Univ. of Penn.*, xvi, p. 143, 1903.

[2] Franchini: Ricambio materiale in acromegalia, *Biol. Scien. f. Med. Bologna Ann.* 75, Ref., *Bioch. Centralbl.* p. 522, 1905.

[3] Mendel: Ref., *Deutsch. med. Woch.*, p. 1975, 1906.

[4] Tauszk and Vas: *Pertei. Med. Chic. Presse*, 1899, Ref. *Jahrb. f. Neurol.*, 1899.

[5] Oberndorfer, E.: Ueber den Stoffwechsel bei Akromegalie, *Zeitschr. f. klin. Med.*, 1908, lxv, p. 6.

It is natural that the reports of the clinical observations should have stimulated a number of experimental investigations on animals on the influence of the pituitary body on the character of the general metabolism.

Oswald[1] experimented on a dog and failed to establish any definite peculiarities in the phosphoric acid output after the administration of the powdered pituitary body. Malcolm[2] also experimented on dogs and noted the following: After administration of the anterior lobe in dried form there occurred a retention of nitrogen and of phosphoric acid and a loss of calcium and magnesium. The administration of the posterior lobe resulted in a retention of nitrogen and a loss of phosphoric acid, followed by a retention of calcium. There was no loss of magnesium. After feeding the fresh gland, the character of the nitrogen and of calcium balance was reversed when compared with that after feeding of the dried gland. The author made the assumption that the destruction of some active substance takes place in the process of drying the gland.

Thompson and Johnston[3] fed dogs on glands dried at 45-60°C. and noted an increased output of nitrogen and of urea, and, in a smaller degree, of phosphoric acid. Very noteworthy was their observation that the administration of glands of young animals caused a more marked change in the metabolism.

Franchini[4] experimented on rabbits and guinea pigs. He injected intravenously sterile extracts of the pituitary body of cattle and of the horse. He noted a marked loss in calcium, magnesium and phosphoric acid output, following the treatment.

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[1] Oswald, A.: Die Chemie und Physiologie des Kropfes, *Virchow's Archiv*, clxix, p. 444.

[2] Malcolm, J.: On the Influence of Pituitary Gland Substance on Metabolism, *Journ. of Physiol.*, xxx, p. 270.

[3] Thompson, W. H. and Johnston, H. M.: Note on the Effects of Pituitary Feeding, *Journ. of Physiol.*, xxxiii, p. 189.

[4] Franchini, G.: Die Funktion der Hypophyse und die Wirkungen der Injektion ihres Extractes bei Tieren, *Berl. Klin. Woch.*, 1910, pp 613, 670, 719.

Mochi[1] experimented on rabbits, injecting subcutaneously suspensions of the gland. He observed a slight loss of nitrogen, a very marked one of phosphoric acid and of calcium. The authors attributed the changes to the destruction of osseous tissue caused by the treatment.

A review of the work of the various observers strikes one by the lack of uniformity in the results of their observations and by the lack of harmony in their conclusions. The cause for this may be found in the fact that the clinical observations were made on patients kept on different diets, on patients in different stages of the disease, and under treatment with the pituitary body prepared in various ways. The influence of the peculiarities of every experiment were not sufficiently considered in the interpretation of the results obtained through it.

The present investigation was carried out on a patient with acromegaly in its resting stage, complicated with glycosuria.

In view of the fact that the complication could obscure the character of the metabolism conditioned by acromegaly it was concluded to direct the first attention on the abatement of the complication. This offered the opportunity to study the peculiarities of acromegaly-glycosuria. It was further planned to make an attempt to ascertain the connection between the course of the glycosuria and the function of the pituitary body. It was hoped to ascertain this connection by testing the carbohydrate tolerance of the patient before and after treatment with extracts of the various parts of the hypophysis.

In relation to the study of the peculiarities of uncomplicated acromegaly attention was directed to the nitrogen and calorific requirement for the purpose of maintaining the nitrogenous equilibrium, and especially towards the peculiarities of the mineral metabolism. It was realized, that in this disease, noted for its very slow progress, the daily deviation of the metabolism from the normal may be too insignificant to be detected by the existing methods of analysis. This consideration made it desirable to compare the peculiarities of metabolism in the normal course of the disease and after administration of the extract from the various parts of the hypophysis.

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[1] Mochi, A.: Il ricambio dell N, P e Ca nei coneli trattati con inietioni di estratto di ipofisi, *Riv. di Pat. rev. e ment*, xv, p. 457, 1910.

Of the two principal theories on the pathogenesis of acromegaly one tended to interpret the symptoms of the disease by exaggerated function of the gland and the other by a depression in its activity. If the first assumption is correct, the peculiarities of the acromegaly metabolism should be intensified by the administration of the gland-extract, in the other case, there should be observed a tendency towards a return to the normal metabolism after treatment with the same extract.

The extract employed in this experiment was prepared by Drs. Sachs and Beebe, in the following manner: The anterior lobes of five glands, freed from connective tissue, were ground up with the addition of sterile physiological salt solution in a sterile mortar. The mixture was made up to a volume of 100 cc. and filtered through a Berkfeld filter. The filtrate was kept in sterile flasks. It had a clear, nearly colorless appearance and contained 0.2 per cent nitrogen. The extract was kept in the refrigerator. The fresh extract was employed in the experiments. (The glands were examined microscopically previously to their use for the extracts.) Two observations were made on the patient. During each observation the patient received 0.2 cc. of the extract on the first day of the experiment, and 0.4 cc. on the second.

The injections were followed by a number of distressing symptoms. There developed a transitory rise of temperature with an increase in the pulse rate. The patient suffered from headache, showed loss of appetite. No local reaction at place of injections could be detected.

History of the patient.

The patient Albert M. . . . native of Russia, 28 years of age, married, tailor by occupation. No excesses in the use of alcohol or tobacco, no record of any specific disease. At the age of 12 received an injury in the back, following which developed epileptic attacks. They continued until the age of 18. They ceased then, and at the same time the increase in size of the fingers and of the feet became apparent to the patient. Later also the enlargement of the jaw became evident. In April of this year, patient developed symptoms of diabetes. Because of these entered the hospital.

Changes in skeleton excepted, patient presented no abnormal physical symptoms.

Wassermann and Noguchi tests negative.

Methods of Analysis.

Analysis was made of urine, feces, and food stuffs.[1]

Nitrogen was estimated according to the Kjeldahl-Gunning method.

Ammonia by the Folin-Shaffer method.

Acetone was estimated volumetrically.

Glucose was determined by Fehling's solution in the usual manner. The cuprous oxide being filtered off and estimated volumetrically. The values for sugar estimated according to Allihn's tables.

Total Ash as sulphates.

Chlorides after Volhard. Feces and food staffs were previously charred with sodium carbonate.

Total Sulphur as barium sulphate after previous fusion in a mixture of sodium hydrate (prepared from sodium) and of potassium nitrate. Gasoline flame was used.

Phosphoric Acid was estimated in the urine volumetrically, potassium ferrocyanide being used as indicator. In feces and food stuffs gravimetrically, after previous fusion in the same manner as for sulphuric acid estimation.

Sodium and Potassium as chlorides from the sulphates.

Calcium gravimetrically as the oxide.

Magnesium, gravimetrically as pyrophosphate.

The results of the observations are recorded in the following tables. On the so called antidiabetic ward diet the daily output of sugar by the patient fluctuated between 100 and 150 grams. After the reduction of the carbohydrate intake to 30–45 grams per day with a simultaneous increase in the fat intake, the symptoms of glycosuria gradually disappeared, and after a short time the tolerance of the patient for carbohydrates increased, so that he could be maintained on a diet containing over 100 grams of carbohydrates, and stood a test of 165 grams carbohydrate intake without developing any symptoms of glycosuria. The patient remained on approximately the same diet through the time of treatment with the gland extract and did not develop any symptoms of glycosuria, although the treatment was followed by a series of unpleasant symptoms. Thus these observations harmonize with the view of those authors who consider glycosuria as an accidental occurrence, not resulting from any faulty secretion of the pituitary body. It is unfortunate that the symptoms following the injections of the extract were so unpleasant to the patient, that it was impossible to test the influence of the extract of the other parts of the gland.

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[1] With the exception of the mineral analysis of the drinking water, of rice, of potato, of butter and of eggs. The values for these were calculated on the basis of the data in König's *Textbook*. The data of the composition of the drinking water was kindly furnished to us by Dr. Atkinson, of the New York Board of Health.

Regarding the nitrogen metabolism and the distribution of the nitrogenous substances in the urine there were comparatively few peculiarities during the natural course of the disease. The nitrogen requirement was comparatively high, since it was difficult to establish in the patient a condition of nitrogenous equilibrium on a diet containing less than 17 grams of nitrogen per day. The intake during practically the entire time of the observation was equivalent to 35 Calories per kilo weight of the patient. Under the influence of the gland extract treatment the nitrogen output of the patient exceeded his intake on the diet which sustained him previously in a state of equilibrium. Following the second injection of the extract, the patient was unable to take his usual diet, and this resulted in a still higher negative nitrogen balance, than after the first injection.

Quite striking are, however, the results of the analysis of the mineral metabolism.

Phosphoric Acid. In the period preceeding the treatment with gland a tendency towards retention of phosphoric acid was observed, After treatment with the extract the output of phosphoric acid increased, bringing about a loss of the substance. In the interval between the two periods of treatment, there was noted a tendency towards reëstablishment of an equilibrium in the output of the substance.

Calcium. Also in regard to this substance there was noted a retention in the period previous to the treatment, and a loss immediately following the treatment.

Magnesium. It was not possible to obtain exact data regarding the intake, but the change in the output of magnesium after treatment made it justifiable to assume that there was a loss of this substance.

Sulphuric Acid. The output of this substance showed an approach to equilibrium (slight retention) in the period precedin the treatment and a loss following it.

Potassium. With one single exception a varying retention o this substance was observed in course of all the observations.

Sodium and Chlorine, showed parallelism in their output. Th balance was negative during the periods of treatment and approac an equilibrium in the intervals.

Whether or not the changes in the mineral metabolism effecte by the injection of the extract resulted from a specific action or from the general rise of metabolism cannot be ascertained wit absolute certainty. From a consideration of the ratios of th nitrogen output to the output of individual mineral substance during the period preceding the first treatment, the impression i gained that the changes in the salt output were not produced merel by the increase in the general metabolism.

Thus the ratios in the normal period were as follows:

$$\frac{N}{\text{Tot. Ash}} \quad \frac{N}{Cl} \quad \frac{N}{SO_3} \quad \frac{N}{P_2O_5} \quad \frac{N}{Na} \quad \frac{N}{K} \quad \frac{N}{CaO}$$

$$\frac{17}{20}=0.85 \quad \frac{17}{6.48}=2 \quad \frac{17}{2.5}=7 \quad \frac{17}{2.86}=6 \quad \frac{17.3}{4.33}=4 \quad \frac{17}{2.33}=7 \quad \frac{17}{1.24}=1$$

Calculating on the basis of these ratios the expected output mineral substances on a nitrogen output of 22.6 grams and compa ing the figures with those actually obtained on analysis, the follo ing data are obtained:

	[illegible]	Cl	SO_3	P_2O_5	Na	K	CaO
Calculated . .	[illegible]	11.3	3.23	3.56	4.60	3.23	1.
Found	[illegible]	9.89	4.89	4.22	5.61	3.39	1.
		-1.41	-1.66	-0.42	-1.00	-0.16	-0.

This table shows that the output of ali mineral constituen with the single exception of that of chlorine exceeded the ca culated values. The chlorine retention might have been cause by the rise of the body temperature.

Following the second injection there was practically no rise i the nitrogen output over the preceding period, though the balanc was negative;—the output of other mineral substances calculated on the basis of the ratio of the period preceding the first treatment does not show the same high values as after the first injection.

	ASH	Cl	SO_3	P_2O_5	Na	K	CaO
Calculated..................	25.4	10.8	3.08	3.60	5.40	3.0	1.54
Found......................	29.4	9.18	3.08	3.50	4.37	3.24	1.46
	−4.0	+0.62	0	+0.10	+1.03	−0.24	+0.08

Thus during this period only the chlorine output markedly exceeded the value calculated for a nitrogen output of 21.6 grams. Of course it is necessary to bear in mind that during this period the intake of the patient was very low.

SUMMARY.

1. In the present case of acromegaly complicated with glycosuria, the latter symptom followed the usual course of glycosuria.
2. The carbohydrate tolerance was in no way affected by the injections of the extract of the anterior lobe of the hypophysis.
3. Following the injection of the same extract there was noted a general rise of metabolism.
4. Following the same injection there were noted peculiarities in salt metabolism, which could not be interpreted on the basis of the rise of the general metabolism.

The clinical observations were made by Dr. S. Wachsman, Medical Director of the Hospital and by his assistants. The authors wish to express their appreciation of the interest taken by him in this work. The authors also wish to acknowledge their indebtedness to Miss Cecil Silverquite for her assistance in preparing the diet charts.

Sulphuric Acid. The output of this substance showed an approach to equilibrium (slight retention) in the period preceding the treatment and a loss following it.

Potassium. With one single exception a varying retention of this substance was observed in course of all the observations.

Sodium and Chlorine. showed parallelism in their output. The balance was negative during the periods of treatment and approach an equilibrium in the intervals.

Whether or not the changes in the mineral metabolism effected by the injection of the extract resulted from a specific action, or from the general rise of metabolism cannot be ascertained with absolute certainty. From a consideration of the ratios of the nitrogen output to the output of individual mineral substances during the period preceding the first treatment, the impression is gained that the changes in the salt output were not produced merely by the increase in the general metabolism.

Thus the ratios in the normal period were as follows:

$$\frac{N}{\text{Tot. Ash}} \quad \frac{N}{Cl} \quad \frac{N}{SO_3} \quad \frac{N}{P_2O_5} \quad \frac{N}{Na} \quad \frac{N}{K} \quad \frac{N}{CaO}$$

$$\frac{17}{20}=0.85 \quad \frac{17}{8.48}=2 \quad \frac{17}{2.5}=7 \quad \frac{17}{2.86}=6 \quad \frac{17.3}{4.33}=4 \quad \frac{17}{2.33}=7 \quad \frac{17}{1.24}=14$$

Calculating on the basis of these ratios the expected output of mineral substances on a nitrogen output of 22.6 grams and comparing the figures with those actually obtained on analysis, the following data are obtained:

	Ash	Cl	SO_3	P_2O_5	Na	K	CaO
Calculated....................	27.0	11.3	3.23	3.86	4.60	3.23	1.60
Found..........................	27.8	9.89	4.89	4.22	5.61	3.39	1.72
		+1.41	−1.66	−0.42	−1.00	−0.16	−0.12

This table shows that the output of all mineral constituents with the single exception of that of chlorine exceeded the calculated values. The chlorine retention might have been caused by the rise of the body temperature.

Following the second injection there was practically no rise in the nitrogen output over the preceding period, though the balance was negative;—the output of other mineral substances calculated on the basis of the ratio of the period preceding the first treatment does not show the same high values as after the first injection.

	ASH	Cl	SO_3	P_2O_5	Na	K	CaO
Calculated....................	25.4	10.8	3.08	3.60	5.40	3.0	1.54
Found..........................	29.4	9.18	3.08	3.50	4.37	3.24	1.46
	−4.0	+0.62	0	+0.10	+1.03	−0.24	+0.08

Thus during this period only the chlorine output markedly exceeded the value calculated for a nitrogen output of 21.6 grams. Of course it is necessary to bear in mind that during this period the intake of the patient was very low.

SUMMARY.

1. In the present case of acromegaly complicated with glycosuria, the latter symptom followed the usual course of glycosuria.

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The clinical observations were made by Dr. S. Wachsman, Medical Director of the Hospital and by his assistants. The authors wish to express their appreciation of the interest taken by him in this work. The authors also wish to acknowledge their indebtedness to Miss Cecil Silverquite for her assistance in preparing the diet charts.

TABLE I.

DATE, JULY 1910	URINE	SPEC. GRAV.	FECES*			T
	cc		gms.			
7–9	1082	1.023	11.6	Intake		
				Output	Urine	
					Feces	
					Sum	
				Balance		+
10–14	1152	1.028		Intake		
				Output	Urine	
					Feces	
					Sum	
				Balance		+
15–17‡	1483	1.025	33.0	Intake		
				Output	Urine	
					Feces	
					Sum	
				Balance		-
18–20	1433	1.027	10.3	Intake		
				Output	Urine	
					Feces	
					Sum	
				Balance		-
21–23‡	1463	1.023	17.0	Intake		
				Output	Urine	
					Feces	
					Sum	
				Balance		-
24–26	1307	1.025	18.7	Intake		
				Output	Urine	
					Feces	
					Sum	
				Balance		-

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* All the figures excepting those in this column represent daily averages.

† 1.5 grams of this amount were given in an enema.

‡ Experimental period.

TABLE I.—Continued.

TOTAL ASH	Cl	TOTAL SO₃	TOTAL P₂O₅	Na	K	CaO	MgO
gms.	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*
	6.22	2.69	2.91	3.40	3.216	1.689	
14.16	5.84	2.18	2.45	2.86	1.86	0.830	0.180
1.06		0.24	0.34	—	0.199	0.339	0.082
		2.42	2.79		2.059	1.169	0.262
	+0.38	*+0.27*	*+0.12*	*+0.54*	*+1.157*	*+0.52*	
	6.13	2.66	2.85	3.43	4.429	1.451	
19.23	8.48	2.305	2.52	4.33	2.137	0.903	0.205
1.06		0.240	0.34	—	0.199	0.339	0.082
		2.545	2.86		2.336	1.242	0.287
	−2.35	*+0.115*	*−0.01*	*−0.90*	*+2.093*	*+0.209*	
	6.20	2.878	3.393	3.644	6.021†	1.483	
24.38	9.70	2.99	3.370	5.480	2.675	1.120	0.256
3.48	0.19	1.90	0.852	0.136	0.724	0.592	0.180
	9.89	4.89	4.222	5.616	3.399	1.712	0.436
	−3.69	*−1.912*	*−0.829*	*−1.972*	*+2.622*	*−0.229*	
	6.20	2.878	3.393	3.644	4.521	1.485	
20.60	7.90	3.12	3.340	4.50	2.396	1.05	0.228
1.23		0.168	0.211	—	0.216	0.20	0.050
		3.288	3.551		2.612	1.25	0.278
	−1.70	*−1.41*	*−0.158*	*−0.856*	*+1.909*	*+0.235*	
	4.91	2.302	2.766	2.776	3.136	1.356	
27.32	9.18	2.744	3.030	4.282	3.01	1.123	0.192
2.11		0.341	0.477	0.097	0.235	0.341	0.132
		3.085	3.507	4.379	3.245	1.464	0.324
	−4.27	*−0.783*	*−0.731*	*−1.603*	*−0.109*	*−0.108*	
	4.99	2.514	2.071	2.845	3.152	1.432	
12.12	2.82	3.568	3.788	0.827	2.56	1.218	0.229
2.31		0.253	0.496	0.117	0.238	0.466	0.149
		3.821	4.284	0.944	2.798	1.684	0.378
	+2.17	*−1.307*	*−2.213*	*+1.891*	*+0.354*	*−0.252*	

TABLE I.

DATE, JULY 1910	URINE	SPEC. GRAV.	FECES*			TOTAL N
	cc		gms.			gms.
7-9	1082	1.023	11.6	Intake		19.52
				Output	Urine	15.31
					Feces	0.71
					Sum	16.02
				Balance		*+3.5*
10-14	1152	1.028		Intake		19.22
				Output	Urine	16.12
					Feces	0.71
					Sum	16.83
				Balance		*+2.39*
15-17‡	1483	1.025	33.0	Intake		19.19
				Output	Urine	20.52
					Feces	2.14
					Sum	22.66
				Balance		*−3.47*
18-20	1433	1.027	10.3	Intake		19.29
				Output	Urine	20.85
					Feces	.71
					Sum	21.56
				Balance		*−2.27*
21-23‡	1463	1.023	17.0	Intake		14.78
				Output	Urine	20.45
					Feces	1.11
					Sum	21.56
				Balance		*−6.78*
24-26	1307	1.025	18.7	Intake		16.76
				Output	Urine	24.10
					Feces	0.84
					Sum	24.94
				Balance		*−7.78*

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* All the figures excepting those in this column represent daily averages.
† 1.5 grams of this amount were given in an enema.
‡ Experimental period.

TABLE I.—Continued.

TOTAL ASH	Cl	TOTAL SO_3	TOTAL P_2O_5	Na	K	CaO	MgO
gms.	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*
	6.22	2.69	2.91	3.40	3.216	1.689	
14.16	5.84	2.18	2.45	2.86	1.86	0.830	0.180
1.06		0.24	0.34	—	0.199	0.339	0.082
		2.42	2.79		2.059	1.169	0.262
	+0.38	*+0.27*	*+0.12*	*+0.54*	*+1.157*	*+0.52*	
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	−1.70	*−1.41*	*−0.158*	*−0.856*	*+1.909*	*+0.235*	
	4.91	2.302	2.766	2.776	3.136	1.356	
27.32	9.18	2.744	3.030	4.282	3.01	1.123	0.192
2.11		0.341	0.477	0.097	0.235	0.341	0.132
		3.085	3.507	4.379	3.245	1.464	0.324
	−4.27	*−0.783*	*−0.731*	*−1.603*	*−0.109*	*−0.108*	
	4.99	2.514	2.071	2.845	3.152	1.432	
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2.31		0.253	0.496	0.117	0.238	0.466	0.149
		3.821	4.284	0.944	2.798	1.684	0.378
	+2.17	*−1.307*	*−2.213*	*+1.891*	*+0.354*	*−0.252*	

TABLE II.

Diet. Daily intake, July 1910.

	7–9	10–14	15–17	18–20	21–23
	cc.	*cc.*	*cc.*	*cc.*	*cc.*
Water, Tea, Coffee	1860	1770	1630	1770	1660
Sour Cream	540	390	390	390	440
Sweet Cream	270	270	180	180	180
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Bread	100	100	100	100	87
Beef	160	160	210	210	123
Beef Cubes	4	4	4	4	2.7
Eggs	96	98	188	188	156
Butter	56	76	40	40	43
Rice	25	25	25	25	17
Cucumbers	100	100	100	100	67
Potatoes		200	200	200	130
NaCl	3	3	3	3	2
Calories	2970	3000	2870	2880	2580

TABLE III.

Mineral Composition of the food.

(Grams in parts per hundred.)

	Cl	SO_3	P_2O_5	Na	K	C
Water*	0.000215	0.000351		0.000267	0.000207	0.0
Sour Cream†	0.096	0.087	0.209	Traces	0.304	0.1
Sweet Cream†	0.08	0.037	0.131	0.014	0.108	0.1
Beef†	0.094	0.435	0.49	0.116	0.379	0.0
Bread†	0.58	1.32	Traces	0.039	0.433	0.1
Beef Cubes†	37.8	0.73	1.97	21.66	3.29	0.4
Cucumbers†	1.342	0.039	0.044	0.775	0.144	0.0
Eggs‡	0.0962	0.0342	0.426	0.1808	0.155	0.1
Rice‡	0.0086	0.0086	0.406	0.49	1.45	0.0
Potatoes‡	0.026	0.0496	0.126	0.0207	0.466	0.0

* Analysed by Board of Health of New York.
† Analysed by Authors.
‡ Calculated from König.

ON THE PRESENCE OF HISTIDINE IN PIG THYREO-GLOBULIN.

By FRED C. KOCH.

(*From the Laboratory of Biochemistry of the University of Chicago.*)

(Received for publication, February 8, 1911.)

Nürnberg[1] in his studies on thyreoglobulin from beef thyroids, carried out the usual Kossel method for the quantitative separation of the hexone bases. He was able to identify arginine and lysine, but stated that he did not obtain enough histidine dichloride crystals to definitely establish its presence.

Following essentially the same methods in a study of hog thyreoglobulin I was able to separate histidine dichloride in sufficient quantities to identify it. The thyreoglobulin was extracted from the dried, almost fat-free hog thyroids[2] by 0.75 per cent sodium chloride solution, precipitated by an equal volume of saturated ammonium sulphate solution. The precipitate was redissolved and reprecipitated thus three times; again dissolved in water, dialyzed until free from sulphate, and finally precipitated by two volumes of 95 per cent alcohol. On hydrolysis and analysis by the Kossel-Osborne methods the following results were obtained from the Kjeldahl nitrogen determinations in the various fractions.

	NÜRNBERG'S RESULTS ON BEEF THYREO-GLOBULIN.	RESULTS ON PIG THYREO-GLOBULIN.
	Per cent of the total nitrogen	*Per cent of the total nitrogen*
Ammonia Nitrogen	5.27	5.14
Arginine Nitrogen	8.71	11.7
Histidine Nitrogen	4.92	5.14
Lysine Nitrogen	12.33	12.57

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[1] *Biochem. Zeitschr.*, xvi, p. 87, 1909.

[2] The raw material for this work was furnished by the Armour Laboratory Department.

No attempt was made to compare the weight of histidine dichloride crystals obtained with the amount calculated from the nitrogen determination on an aliquot part of the histidine fraction. These crystals were the characteristic prisms arranged in clusters, gave a very strong diazo-reaction, and after recrystallizing three times from concentrated hydrochloric acid, melted[1] at 233–235° C. Nitrogen estimations by the Kjeldahl method gave low results, but so also does pure histidine dichlorid eobtained from beef hemoglobin, as the following figures show:

	PER CENT NITROGEN
Crystals (recrystallized three times)	17.55
Crystals (recrystallized four times)	17.45
Histidine dichloride from beef hemoglobin	17.78
Theoretical for $C_6H_9N_3O_2 2\ HCl$	18.44

I wish to express my thanks to Prof. A. P. Mathews for suggestions and interest in this work.

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[1] Kutscher (*Zeitschr. f. physiol. Chem.*, xxviii, p. 383) gave the melting point as 231–233°C.

THE FATE OF BENZOYLACETIC ACID IN THE ANIMAL BODY.

By H. D. DAKIN.

(From the Herter Laboratory, 819 Madison Avenue, New York.)

(Received for publication, February 11, 1911.)

Studies upon the fate of the phenyl derivatives of fatty acids have thrown much light upon the possible transformation which the naturally occurring fatty acids may undergo in the course of their catabolism. Of these investigations of phenyl derivatives, that upon phenylpropionic acid has been particularly illuminating. By injecting cats with the sodium salt of this acid, it has been possible to determine the formation of the following intermediate substances in addition to the end product of oxidation, benzoic acid, which is excreted in the form of hippuric acid:[1]

Phenyl-β-hydroxy-propionic	$C_6H_5.CHOH.CH_2.COOH$
Cinnamoyl-glycocoll	$C_6H_5.CH{:}CH{:}CO.NH.CH_2.COOH$
Benzoylacetic acid	$C_6H_5.CO.CH_2.COOH$
Acetophenone	$C_6H_5.CO.CH_3$
Hippuric acid	$C_6H_5.CO.NH.CH_2.COOH$

The relationships between these substances are somewhat complicated but they furnish a very striking demonstration of the essential accuracy of Knoop's hypothesis of the oxidation of fatty acids at the β-position.[2]

The relationship between acetophenone and benzoylacetic acid is evidently similar to that between acetone and aceto-acetic acid and all the evidence available tends to the belief that the ketones are derived from the ketonic acids by an *irreversible reaction.* In the case of the other substances the relationships are more complex. The unsaturated cinnamic acid readily passes over in part into phenyl-β-hydroxypropionic acid,[1] while administration of salts of phenyl-β-hydroxypropionic may result in the excretion of cinnamic acid derivatives. The change here is evidently a *reversible* one:

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[1] This *Journal*, iv, p. 419, 1908; v, p. 303, 1908; vi, p. 203, 1909.

[2] Knoop: *Beitr. z. physiol. u. path. Chem.*, vi, p. 155, 1904.

$$C_6H_5.CH{:}CH.COOH \overset{\pm H_2O}{\rightleftarrows} C_6H_5.CHOH.CH_2.COOH$$

It has been shown that both cinnamic acid and phenyl-β-oxypropionic acid when administered as salts to cats may yield benzoylacetic acid (and hence acetophenone).

$$\begin{tikzcd}
C_6H_5.CH{:}CH.COOH \arrow[rr] \arrow[dr] & & C_6H_5.CHOH.CH_2.COOH \arrow[dl] \\
& C_6H_5.\ CO\ .\ CH_2\ .\ COOH &
\end{tikzcd}$$

It appeared not improbable that these reactions might also prove to be *reversible*, especially in view of the fact that Blum,[2] Dakin,[3] Wakeman,[4] Friedmann and Maase[5] have recently independently demonstrated the formation of *l*-β-hydroxybutyric acid by the asymmetric reduction in the body of aceto-acetic acid.

Experiments made a year ago with the object of determining this question showed that the reaction was in fact reversible. Sodium benzoylacetate when given intravenously or subcutaneously to cats in addition to yielding hippuric acid as observed by Knoop, in part was transformed into lævo-rotatory phenyl-β-hydroxypropionic acid and cinnamoylglycocoll. In the meantime Friedmann[6] arrived at the same conclusion although he did not succeed in isolating the β-hydroxy-acid. He obtained a lævo-rotatory acid soluble in ether which gave cinnamic acid on heating with hydrochloric acid. These results might equally well be due to the corresponding glycocoll derivative, $C_6H_5.CHOH.CH_2.CO.NH.CH_2.COOH$, the synthesis of which has been described in this Journal.[7] Since the writer had been successful in actually isolating the pure lævo-rotatory phenyl-β-hydroxypropionic acid and found its properties to agree with those of the active acid resolved by McKenzie the results appeared worthy of record.

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[1] This *Journal*, vi, p. 203, 1909.

[2] *Munch. med. Woch*,. No. 13, 1910.

[3] This *Journal*, viii, p. 97, 1910.

[4] This *Journal*, viii, p. 105, 1910.

[5] *Biochem. Zeitschr.* xxvii, p. 474, 1910.

[6] *Biochem. Zeitschr.* xxvii, p. 119, 1910.

[7] This *Journal*, v, p. 303, 1908.

The details of the experiments are given in the following pages. The yield of phenyl-β-oxypropionic acid was relatively large; that of cinnamoylglycocoll was considerably less. Considerable amounts of unchanged benzoylacetic acid and acetophenone were always found in the urines.

In picturing the possible reactions concerned in the oxidation of phenylpropionic acid to benzoic acid in the animal body, it will therefore be necessary to take cognizance of the possible reversible interconversion of phenyl-β-hydroxybutyric acid and benzoylacetic acid. The following scheme is in harmony with the facts so far as at present ascertained.

$$\begin{tikzcd}
 & C_6H_5.CH_2.CH_2.COOH \arrow[dl] \arrow[d] & \\
\textit{lævo}\text{-}C_6H_5.CHOH.CH_2.COOH \arrow[d, shift left] & C_6H_5.CO.CH_2.COOH \arrow[r] \arrow[d] & C_6H_5.CO.CH_3 \arrow[dl] \\
C_6H_5.CH:CH.COOH \arrow[u, shift left] \arrow[r] \arrow[d] & C_6H_5.COOH \arrow[d] & \\
C_6H_5.CH:CH.CO.NH_2CH_2.COOH & C_6H_5.CO.NH.CH_2.COOH &
\end{tikzcd}$$

The experimental part of this paper also contains an account of the demonstration of the excretion of cinnamoylglycocoll when a solution of sodium cinnamate together with glycocoll was slowly injected into the femoral vein of cats. The amount of this substance recovered was, however, surprisingly small.

EXPERIMENTAL PART.

Benzoylacetic ethyl ester was prepared according to Claisen's directions by the limited action of ammonia upon the sodium salt of benzoyl-aceto-acetic ester. The reaction proceeds smoothly and gives an excellent yield of the ester b.p.155-160° at 16mm. pressure. This method is decidedly more convenient than any of the older methods of preparation.

Benzoylacetic acid was prepared by the saponification of the ester as follows: 19.2 grams of the ester were shaken with 102 cc. of cold normal sodium hydrate solution. After standing for twenty-four hours, the solution was filtered from a little oil and acidified with sulphuric acid. An abundant precipitate of crystalline benzoylacetic acid was obtained and an additional quantity was recovered from the mother liquor by extraction with ether. The yield of acid was 14 grams.

Three experiments were made to determine the fate of benzoylacetic acid in the body. In two of these 2.0 and 2.5 grams of the acid were neutralized with caustic soda and injected subcutaneously into cats. In the third experiment the sodium salt corresponding to 3 grams of the acid was injected slowly in dilute aqueous solution into the femoral vein of a cat. From the rotations of the ether extract of the urines it was calculated that the amount of β-hydroxy-phenylpropionic acid excreted in the urine amounted to 0.8, 1.1, 0.5 gram respectively. The methods of urinary analysis were the same in each case so that a general description will suffice.

In each case a portion of the urine was acidified and shaken with ether. The ethereal extract was dissolved in a little alcohol and tested with ferric chloride. A positive reaction for unchanged benzoylacetic acid was invariably obtained. Another portion of the urine was distilled with phosphoric acid. The distillate gave the typical reactions for acetophenone strongly and on adding an acetic acid solution of *p*-nitro-phenylhydrazine, a sparingly soluble hydrazone was at once precipitated, which after recrystallization from dilute alcohol melted at 184-185°, corresponding to acetophenone *p*-nitro-phenylhydrazone.

The remaining urine was acidified with phosphoric acid and extracted in a continuous extractor with ether. The ethereal extract was distilled in steam to remove volatile fatty acids, clarified with charcoal and the concentrated solution examined in the polarimeter. A strong laevo-rotation was observed in each case corresponding to the amounts of *l*-phenyl-β-hydroxy-phenylpropionic acid previously recorded. The hydroxy-acid was separated as follows: The solution was concentrated to small volume and allowed to crystallize. The crystals consisting of a mixture of cinnamoylglycocoll and hippuric acid were filtered off and reserved. The filtrate was twice shaken out with ether. The ethereal extract on evaporation left a chalky mass of the crude hydroxy-acid contaminated with some hippuric acid. The hydroxy-acid was separated by extraction with boiling chloroform but did not crystallize on evaporation of the solvent. On dissolving in water, however, and allowing the solution to stand a good yield of fine colorless needles of the pure phenyl-β-hydroxypropionic acid was readily obtained. The substance, after recrystallization from boiling toluene melted sharply at 115-116°. Its properties agreed in every respect with those of the laevo-acid recently obtained by McKenzie and Humphries[1] by the resolution of inactive phenyl-β-hydroxypropionic acid.

Analysis: 0.0963 gm. gave 0.2280 gm. CO_2 and 0.0530 gm. H_2O

	Found:	Calculated for $C_9H_{10}O_3$:
Carbon	64.6	65.1
Hydrogen	6.1	6.0

A small amount of the crystals served for the approximate determination of the optical rotation. The value obtained was very close to that determined by McKenzie and Humphries.

$$l=1;\ c=2.65;\ \alpha=0.49^\circ;\ [\alpha]^{20^\circ} = -18.5^\circ.$$

On boiling the crystals for a few minutes with dilute hydrochloric acid and subsequently cooling, an abundant precipitate of cinnamic acid, m.p., 132-133°, was readily obtained.

The crude crystals consisting of a mixture of hippuric acid and cinnamoylglycocoll separated in an early stage of the analysis and previously referred to, amounted to about 1.5 grams from the three experiments. On recrystallizing repeatedly from hot water a small quantity of pure cinnamoylglycocoll m.p., 192–193°, crystallizing in the form of long shining needles was obtained. The substance dissolved in dilute sodium carbonate solution instantly reduced dilute potassium permanganate solution with production of benzaldehyde. The amount of pure substance was not sufficient for analysis.

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[1] *Trans. Chem. Soc.*, xcvii, p. 121, 1910.

Two experiments were made in which a solution containing 3.0 grams of cinnamic acid in the form of its sodium salt and 2.0 grams of glycocoll dissolved in 150 cc. of water was slowly injected into the femoral vein of large cats. The time of injection was between three and four hours and the mixture was evidently toxic. The urines secreted from both cats after the administration of the cinnamate was examined for cinnamoylglycocoll by the methods which have so frequently been made use of previously. In each case between 0.1 and 0.2 gram of cinnamoylglycocoll was separated from the urine. The separation of this substance from accompanying hippuric acid involves considerable loss but taking this into consideration the yield of cinnamoylglycocoll was surprisingly small.

SUMMARY.

Benzoylacetic acid (2-3 grams) administered to cats in the form of its sodium salt either intravenously or subcutaneously results in the excretion in the urine of cinnamoylglycocoll and of lævorotatory phenyl-β-hydroxyphenylpropionic acid. The formation of the latter substance is completely analogous to the asymmetric reduction in the animal body of aceto-acetic acid to *l*-hydroxybutyric acid observed by Blum, Dakin and Wakeman and Friedman and Maase.

The *l*-β-hydroxyphenylpropionic acid was isolated from the urines in the pure state and had properties identical with those of the acid recently obtained by McKenzie and Humphries by the resolution of inactive phenyl-β-hydroxypropionic acid by means of alkaloids.

The experiments throw further light upon the mode of catabolism of phenylpropionic acid. These changes are represented diagramatically on page 125. β-Hydroxyphenylpropionic acid and benzoylacetic acid are mutually interconvertible in the animal body by means of reversible reactions involving oxidation and reduction respectively.

Experiments are described showing that an excretion of cinnamoylglycocoll may follow thae intravenous injection of sodium cinnamate and glycocoll. The yield is poor, however.

CONCERNING NUCLEASES.

By WALTER JONES.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

(Received for publication, February 21, 1911.)

The term *nuclease* is usually understood to designate a ferment, almost or quite universally present in animal glands, through whose agency nucleic acid is decomposed with the liberation of purine bases which thus become directly precipitable by the reagents commonly employed for this purpose. But an examination of the experimental evidence upon which this definition rests will show that it is ambiguous and very unsatisfactory. Jwanoff[1], who first used the term, found that when certain moulds (*Penicillium glaucum* and *Aspergillus niger*) are grown upon thymus nucleic acid, directly precipitable purine bases make their appearance; and as the active agent here concerned was found incapable of liquefying gelatine, Jwanoff concluded that he was dealing not with trypsin but with a ferment specially adapted to the decomposition of nucleic acid. But E. Salkowski[2] had long since proven that in aqueous extracts of various animal glands, free purine bases appear as digestion proceeds at the body temperature. Salkowski's results show that each gland is supplied with an active agent that can effect the decomposition of some nucleic acid present in that particular gland but there is no assurance that the ferment of one gland can decompose the nucleic acid of another gland. Nor does a study of the structure of the nucleic acids indicate such a probability. The recent work of Levene and Jacobs[3] has proven that all of the well known substances belonging to this group are chemically constituted after the same general type; that is to say, in every one of these compounds, a phosphoric acid group is linked to a nitrogenous ring by a group of d-ribose. While this essential "nucleotide" structure is common to all nucleic acids, individual differences in the nitrogenous rings and in reduplication of molecule are so great that one would be somewhat surprised to find one physiological agent that can decompose them all.

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[1] Jwanoff: *Zeitschr. f. physiol. Chem.*, xxxix, p. 31.

[2] E. Salkowski: *ibid.*, xiii, p. 506.

[3] Levene and Jacobs: *Ber. d. d. chem. Ges.*, xli, xlii, xliii.

In order to examine carefully into this question it was determined to make a close study of the behavior of guanylic acid when it is allowed to remain at the body temperature in contact with aqueous extracts of various animal glands which were known to contain the ferment guanase. The reasons for this selection and the justification for the conclusions finally drawn will appear from the following considerations. It is somewhat difficult to decide whether or not a ferment has brought about the decomposition of a nucleic acid unless the decomposition is complete. The methods for the recovery of the undecomposed nucleic acid are so poor that one cannot always decide whether a partial disappearance of the substance is due to ferment action or to analytical error. A search for the decomposition products is also attended with some uncertainty for the precipitation of purine bases with reagents is prevented by the presence of residual nucleic acid, and an attempt to remove this interfering substance might easily result in a removal also of purine bases or in the liberation of purine bases which would be more confusing. The employment of guanylic acid avoids difficulties of this nature. The substance is a mononucleotide which yields only one purine base (guanine) and its presence in a solution does not prevent the precipitation of free purine bases either by copper reagent or by silver reagent.

Gland extracts rich in guanase were chosen because in such extracts guanine is never found as an end product of ferment action. If a ferment be present which splits off guanine from a nucleic acid, the base will be promptly converted into xanthine. Thus one is scarcely concerned with the precautions usually employed in the isolation of the products; in fact the products of digestion may even be submitted to hydrolysis with boiling mineral acid if this be found desirable. No confusion can arise concerning the origin of the products, for guanine always means "acid hydrolysis," never "gland action;" and the finding of guanine without adenine suggests not only "acid hydrolysis," but "acid hydrolysis of guanylic acid."

Proceeding upon these principles it was easily proven that an extract of ox spleen can rapidly effect the decomposition of guanylic acid giving rise to xanthine but that an extract of pig's pancreas cannot. After a comparatively short digestion of guanylic acid with an aqueous extract of ox spleen the product is found rich in xanthine directly precipitable by silver nitrate and ammonia, and corresponding in amount to the guanylic acid employed. The complete disappearance of guanylic acid is shown by the fact that hydrolysis of the products of digestion does not produce guanine. With pigs pancreas the matter is entirely different. At the end of a prolonged digestion of guanylic acid with an aqueous extract of this gland, the product contains only a small amount of purine material that is directly precipitable. This free purine material is evidently not attributable to the added guanylic acid since it contains both xanthine and hypoxanthine and is found as well in a digested gland extract to which no guanylic acid has been added. If now the products of digestion be boiled with sulphuric acid free guanine becomes abundantly present without the simultaneous appearance of even a trace of adenine. Plainly, ox spleen contains a ferment guanylase which decomposes guanylic acid but pig's pancreas does not. The remote possibility that pig's pancreas can split phosphoric acid from guanylic acid, giving rise to guanosin, is ignored until determinations of phosphoric acid can be made.

The result obtained with ox spleen might have been expected; that with pig's pancreas is a little surprising, especially as the specimen of guanylic acid employed had been prepared from pig's pancreas. The little that is known on the subject would lead one to suspect that the pancreas contains a ferment "guanylase;" for in all directions for the preparation of guanylic acid from this gland the reader is warned to use perfectly fresh tissue and to heat the aqueous extract as rapidly as possible to the boiling point. These precautions suggest that guanylic acid disappears from the gland when conditions are brought about which are favorable to autodigestion. In order to test this question an examination was made of the products of autodigestion of pig's pancreas. In accordance with former experiments reported from this laboratory[1] it was found that as autodigestion proceeds, free xanthine and hypoxanthine appear, but neither guanine nor adenine can be found among the products. But if the products of autodigestion be boiled with sulphuric acid, an excessive amount of free guanine appears without the simultaneous appearance of adenine. This suggests that the native guanylic acid of pig's pancreas survives an autodigestion of the gland.

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[1] Jones and Partridge: *Zeitschr. f. physiol. Chem.*, xlii, p. 343.

The results described serve to explain a disputed point. Six years ago when the writer was engaged in a discussion of the independent existence of guanase and adenase an article appeared by Schenck[1] in which it was claimed that in the self-digestion of pig's pancreas, hypoxanthine and guanine are abundantly produced but that neither adenine nor xanthine can be found among the products. This author was good enough to explain that his results pointed clearly to the existence of two desamidases, one of which could not be present in the pancreas. Schenck's findings would have been effectively used in the argument referred to had he obtained his results with some other tissue: but the writer[2] was at the time thoroughly convinced that the pancreas can desamidize guanine and adenine with equal ease. Since the results described in this paperwere obtained, the article of Schenck was again referred to when it wasnoticed that he boiled the autolysed product with mineral acid before determining the purine bases. The guanine which he obtained was not an autolytic product but was produced by acid hydrolysis of the guanylic acid which survived autolysis. How Schenck failed to find xanthine among the products is more difficult to explain for this base is a never failing autolytic product of pig's pancreas.

EXPERIMENTAL PART.

The guanylic acid used in this work was prepared from pig's pancreas by the method which Steudel and Briegl describe for the preparation of the substance from ox pancreas.[3]

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[1] Schenck: *Zeitschr. f. physiol. Chem.*, xliii, p. 406.

[2] Jones and Partridge: *loc. cit.*

[3] Steudel and Briegl: *Zeitschr. f. physiol. Chem.*, lxviii, p. 40.

The gland extracts were made by agitating one part of the trimmed and ground gland with three parts of distilled water in tightly corked bottles which contained a sufficient amount of chloroform to prevent putrefaction, The guanylic acid was added sometimes to the mixture of gland and water. sometimes to an extract that had been strained through linen.

At the end of the digestion the product was boiled and filtered and the purine bases precipitated with copper sulphate and sodium bisulphite. When the product was to be hydrolysed, sulphuric acid was added (2000 cc. of 20 per cent acid per liter of fluid) and the material was boiled briskly for one hour. After neutralizing most of the sulphuric acid with caustic soda, the purine bases were precipitated with copper sulphate and sodium bisulphite. The copper precipitate was decomposed with sodium sulphide and the emulsion broken up with sulphuric acid which was purposely used instead of acetic acid and was added in considerable excess. The yellow filtrate from copper sulphide without neutralizing the sulphuric acid, was treated with copper sulphate and sodium bisulphite and this second copper precipitate decomposed as was the first with sodium sulphide and sulphuric acid. By this substitution of sulphuric acid for acetic acid danger of losing guanine is avoided and in the second precipitation of the copper compounds, coloring matter is held in solution so that the final filtrate from copper sulphide is always colorless. This solution was made strongly alkaline with ammonia and treated with a moderate excess of an ammoniacal solution of silver nitrate. The silver compounds thus precipitated were decomposed with hydrochloric acid, and after evaporation of the filtrate from silver chloride the pale yellow residue was ground with hot water and treated with the proper excess of ammonia for the precipitation of guanine. In cases where guanine was obtained the base was dissolved in very dilute caustic soda, precipitated with acetic acid, suspended in boiling water and brought into solution with the smallest possible amount of sulphuric acid. The pale yellow solution was boiled with a very small quantity of animal charcoal and filtered when a solution was obtained from which ammonia precipitates snow-white guanine. Some of the specimens of guanine were analysed, others were converted into the crystalline chloride which was analysed.

The ammoniacal filtrate from guanine was boiled until free from ammonia, 5 cc. of 20 per cent hydrochloric acid were added and the solution was evaporated *just* to dryness on the water bath. The residue was extracted with 0.5 per cent hydrochloric acid at 60.° By this procedure a very small quantity of xanthine passes into solution but no hypoxanthine remains undissolved which is almost sure to be the case when the separation of the bases is made strictly according to the directions of Krüger and Solomon.

The crude xanthine was purified through its nitrate and the free base analyzed.

The different hydroxanthine fractions were variously dealt with. In no case did a drop of the solution produce an immediate precipitate with picric acid, but occasionally solutions were obtained in which picric acid produced a cloudiness on standing an hour or more. These were products of the combined autolysis and hydrolysis of pig's pancreas where the time of autolysis was short. By treating such solutions with a great excess of picric acid and allowing to stand over night minute quantities of a picrate were formed which on recrystallization appeared as pale yellow silky hairs resembling adenine picrate.

In all cases hypoxanthine was purified by means of its nitrate and analysed as free base. In the purification of this substance quantitative relations were often lost in an attempt to prepare products that gave no response to the xanthine color reaction. The amounts of hypoxanthine however, can have nothing to do with matters under discussion in this paper.

From a large amount of data the following are selected as sufficient to prove the statements of this paper.

1. Ox Spleen and Guanylic Acid.

Strained aqueous extract of the gland (1:3)	300 cc.
Guanylic acid	600 mg.
Equivalent guanine	216 mg.
Chloroform	5 cc.

Digestion at 40°, 3 days.

Bases isolated *without previous acid hydrolysis.*

Xanthine found	254 mg. (N = 37.3 instead of 36.8)
Guanine	absent
Adenine	absent

The crude xanthine contained a small amount of uric acid which was destroyed in the purification of the base through its nitrate.

Strained aqueous extract of the gland (1:3)	300 cc.
Guanylic acid	600 mg.
Equivalent guanine	216 mg.
Chloroform	5 cc.

Digestion at 40°, 2½ days.

Bases isolated *after previous acid hydrolysis.*

Xanthine found	268 mg. (N = 37.00 instead of 36.8)
Guanine	absent
Adenine	absent

Strained extract of this gland (1:3) *boiled*	300 cc.
Guanylic acid	600 mg.
Equivalent guanine	216 mg.
Chloroform	5 cc.

Digestion 3 days at 40°.

Free purine bases present only in very small amount, so that the product was boiled with sulphuric acid.

Guanine found	202 mg. (N = 46.1 instead of 46.4)
Xanthine	traces
Adenine	uncertain traces

Ox spleen contains an active agent, destroyed by heat, through whose agency guanylic acid under goes hydrolysis with liberation of guanine. This free guanine is of course immediately converted into xanthine by the guanase of the tissue.

2. *Pig's Pancreas and Guanylic Acid.*

Ground pancreas	280 mg.
Distilled water	840 cc.
Chloroform	16 cc.
Guanylic acid	1.8 gm.
Equivalent guanine	659 mg.

Digestion of 40°, 6½ days.

The product contained only a small amount of purine material directly precipitable by silver nitrate and ammonia and was therefore boiled with sulphuric acid.

Guanine found	905 mg. (N = 46.30 and 46.4 instead of 46.36)
Guanine of a check test to which no guanylic acid was added	359 mg.
Xanthine found	172 mg.
Xanthine of a check test	165 mg.

As may have been expected there is some loss of guanine (or guanylic acid) but there is no increase of xanthine which corresponds. Adenine was absent. The hypoxanthine fraction was treated with a great excess of picric acid and allowed to stand over night. There was no suggestion of a precipitate, yet a drop of the fluid responded promptly to a drop of 0.25 per cent adenine sulphate.

Strained extract of pancreas	400 cc.
Guanylic acid	500 mg.
Equivalent guanine	230 mg.
Chloroform	7 cc.

Digestion at 40°, 5 days.

The product was hydrolyzed with boiling sulphuric acid before the bases were isolated.

Guanine found	246 mg.
Guanine of a check test	about 10 mg.
Xanthine too small in amount to purify.	
Adenine	absent.

The guanine was converted into the chloride which was analysed

H_2O = 15.94 instead of 16.11
N = 37.10 instead of 37.33.

3. *Pig's Pancreas and Guanine.*

Strained extract of pig's pancreas (1:3)	400 cc.
Guanine chloride (in alkali)	400 mg.
Equivalent guanine	267 mg.
Chloroform	5 cc.

Digestion at 40°, 5 days.

Xanthine found.................294 mg. (N = 36.6 instead of 36.8)

Guanine...absent

Guanase is present in pig's pancreas. This experiment is given to confirm the previous work of Jones and Partridge which was called somewhat into question by Schenck's work on the self digestion of this gland.

4. Pig's Pancreas and Adenine.

Strained extract of pancreas (1:3)	400 cc.
Adenine sulphate (in alkali)	400 mg.
Equivalent adenine	267 mg.
Chloroform	5 cc.

Digestion at 40°, 5 days.

Hypoxanthine nitrate found..........................350 mg.

Equivalent hypoxanthine.....233 mg. (Free base analysed N = 41.4 instead of 41.2)

Adenine ....absent

The presence of adenase in pig's pancreas has been assumed from the conduct of the tissue in self digestion but so far as I can remember the proof is reported here for the first time.

5. Self-digestion of Pig's Pancreas.

Into a number of 4 L. bottles were placed 1 K. of trimmed and ground fresh pancreas with 3 L. distilled water and 60 cc. of chloroform. The vessels were tightly closed and allowed to remain at the body temperature for various intervals of time. As each portion was removed the fluid was boiled and filtered. After removal of the fat with a separating funnel, two portions of the fluid of 3 L. and 1 L. respectively were measured out. In the larger portion the purine bases were directly determined and the numbers divided by three. The smaller portion was hydrolyzed with sulphuric acid before determining the purine bases. The results are given in the table in milligrams: hypoxanthine was not estimated.

TIME OF AUTOLYSIS	AUTOLYSIS ALONE			AUTOLYSIS AND ACID HYDROLYSIS		
	Guanine	Adenine	Xanthine	Guanine	Adenine	Xanthine
17 hours........	0.000	0.000	151	384	8	160
44 hours........	0.000	0.000	160	402	19	161
66 hours........	0.000	0.000	142	386	000	174
168 hours.......	0.000	0.000	155	399	000	169
240 hours.......	0.000	0.000	139	397	000	180

The gland evidently contains a mixed nucleic acid and its corresponding nuclease. Guanine and adenine are liberated as digestion proceeds, but are immediately converted into the corresponding oxypurines by the guanase and adenase present. This reaction appears to be very rapid for the free xanthine at the end of 240 hours is no greater than at the end of 17 hours.

It is exceedingly difficult to estimate both xanthine and hypoxanthine in the same specimen so that the hypoxanthine was sacrificed to xanthine whose determination bears more directly upon the matters here discussed. But hypoxanthine was always found present.

Why the xanthine is slightly but uniformly increased by hydrolysis, I cannot say, but am inclined to believe that its copper compound is slightly more soluble in the fluid that has not been hydrolyzed. The trace of adenine found in the shorter digestions is probably due to inclusions that are broken up as digestion proceeds.

The important matter is the sharp appearance of guanine without adenine when the product is hydrolysed with acid, the amount being independent of the duration of digestion. Thus the native guanylic acid of pig's pancreas survives the autodigestion of the gland.

The following experiment was made before the distribution of ferments in pig's pancreas was known or even suspected and the results seemed scarcely credible. Arranged as a laboratory experiment, it can be employed to compel one through his curiosity to an understanding of nuclein fermentation. A mixture of 500 grams of pig's pancreas with 1500 cc. of distilled water and 30 cc. of chloroform is placed in a tightly closed vessel and allowed to digest at 40.° Material that has been left in the thermostat and forgotten will be well enough. The product is boiled and filtered and, taking the text-book cue, the solution is treated with sulphuric acid and boiled *to remove interfering substances*. The preponderating purine compound is guanine which can easily be isolated and purified. One will obtain about 1.0 gram of guanine after full purification through the chloride. A portion of this guanine is dissolved in caustic soda and digested with a little fresh extract of pig's pancreas for several days at 40°. For the sake of uniformity the product may be boiled with sulphuric acid. Xanthine will be found in great preponderance and guanine will be so small in amount as to altogether escape detection. Thus to all appearances guanine obtained from digested pancreas extract is converted by pancreas extract into xanthine.

THE CATABOLISM OF PHENYLALANINE, TYROSINE AND OF THEIR DERIVATIVES.

By A. J. WAKEMAN and H. D. DAKIN.

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(Received for publication, February 16, 1911.)

The study of alcaptonuria has yielded results of much value upon which to base speculations as to the mode of catabolism of the aromatic amino-acids, phenylalanine and tyrosine, in the normal and alcaptonuric organisms. As is well known these two amino-acids, both of them important derivatives of proteins, are apparently completely catabolized in the normal organism with production of ammonia or urea, carbon dioxide and water; while in the case of the alcaptonuric both the amino-acids yield homogentisic acid. This latter reaction is very remarkable and involves a rearrangement of the relative positions of the side-chain and hydroxyl groups in tyrosine and also the introduction of a second hydroxyl group. This type of change is associated with the intramolecular rearrangement of substances possessing a quinonoid structure.

The inference was natural that a substance of quinonoid structure is the precursor of homogentisic acid.[1] Neubauer[2] as the result of his extensive investigations has indicated the important part which the α-ketonic acids play in representing an intermediate stage between the α-amino-acids and the corresponding fatty acids with one less carbon atom. Embden[3] on the other hand has shown that not only phenylalanine and tyrosine but also homogentisic acid yield aceto-acetic acid and acetone on perfusion through an excised normal liver.

With these basic facts for guidance Neubauer has constructed the following scheme to represent diagrammatically the catabolism of tyrosine and of phenylalanine in the normal organism.

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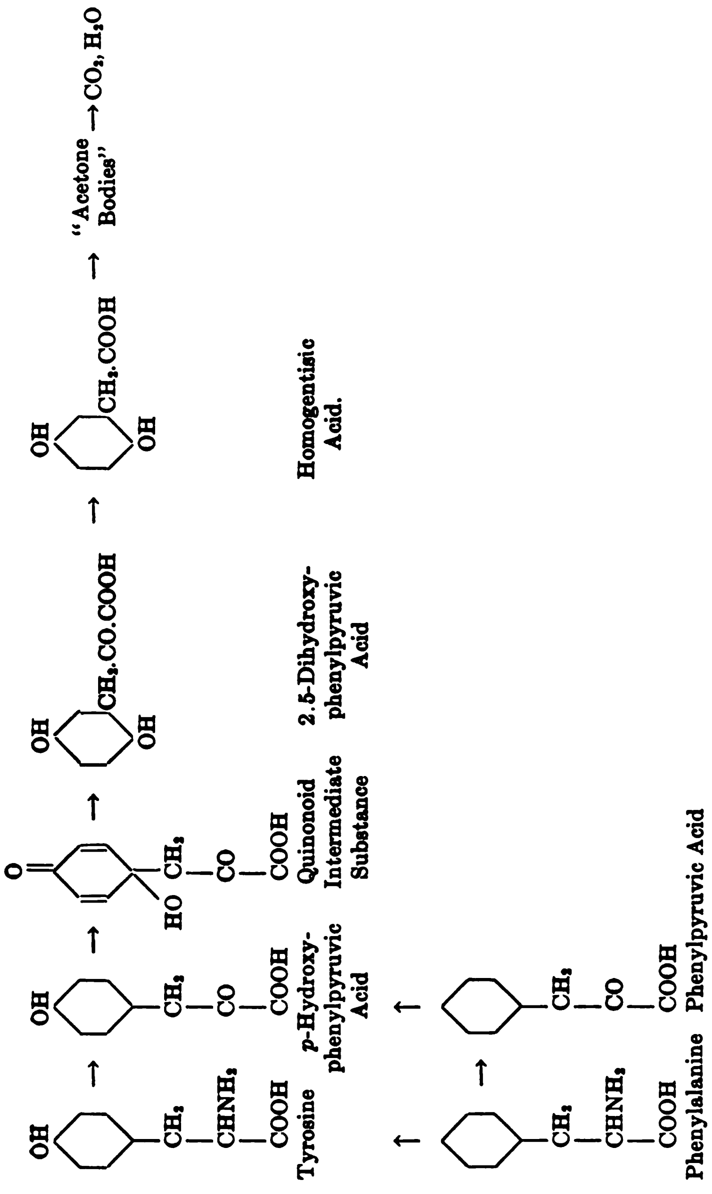
[1] E. Meyer: *Deutsch. Arch. f. klin. Med.*, lxx, p. 447, 1901.

[2] O. Neubauer: *Ibid.*, xc, p. 211, 1909.

[3] *Beitr. z. chem. Physiol. u. Path.*, viii, p. 153, 1906.


OH
CH₂
CHNH₂
COOH
Tyrosine
OH
CH₂
CO
COOH
p-Hydroxyphenylpyruvic Acid
O
HO
CH₂
CO
COOH
Quinonoid Intermediate Substance
OH
CH₂.CO.COOH
OH
2.5-Dihydroxyphenylpyruvic Acid
OH
CH₂.COOH
OH
Homogentisic Acid.
"Acetone Bodies" →CO₂, H₂O
CH₂
CHNH₂
COOH
Phenylalanine
CH₂
CO
COOH
Phenylpyruvic Acid

It will be seen from this diagram that:

(1) Phenylalanine is represented as being converted into tyrosine or into para-hydroxyphenylpyruvic acid (through the stage of phenylpyruvic acid) by introduction of an hydroxyl group in the para-position.

(2) Para-hydroxyphenlypyruvic acid is represented as being converted into a substance of quinonoid structure which by intramolecular rearrangement passes over into 2.5-dihydroxyphenylpyruvic acid; the latter substance yielding homogentisic acid by oxidation.

(3) The homogentisic acid thus formed undergoes decomposition with formation of "acetone bodies" involving disruption of the benzene ring. 'The "acetone bodies" (aceto-acetic acid, acetone and β-hydroxybutyric acid) are then finally oxidized to carbon dioxide and water.

Neubauer, following the majority of other workers[1] *upon this subject, regards homogentisic acid as a normal product of the catabolism of tyrosine and of phenylalanine. Alcaptonuria is regarded as a condition in which there is simply a failure to deal with a normal product of intermediary metabolism, namely homogentisic acid.*

From the results of experiments upon the fate of the derivatives of phenylalanine and of tyrosine in the normal and alcaptonuric organism we are of the opinion that Neubauer's representation of the normal catabolism of phenylalanine and of tyrosine will require considerable modification. The reasons for this opinion are as follows:

(1) No evidence has been put forward that phenylalanine undergoes hydroxylation in the aromatic nucleus under normal conditions. Injections of phenylalanine, so large that much appears unchanged in the urine, fail to evoke any excretion of phenolic substances including homogentisic acid.[2]

(2) It is improbable that either a substance of quinonoid structure such as is represented by Neubauer as a precursor of homogentisic acid or homogentisic acid itself is formed in the course of the normal catabolism of phenylalanine and tyrosine.

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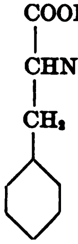
[1] For literature references see This *Journal*, viii, p. 11, 1910.

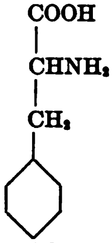
[2] Cf. Dakin: This *Journal*, vi, p. 235, 1909.

The evidence for this is based upon the fact that para-methylphenylalanine and para-methoxy-phenylalanine[1] *(tyrosine-methyl-ether) both substances which by reason of their constitution cannot be converted into para-quinonoid derivatives, undergo practically complete oxidation in the normal organism in precisely the same fashion as phenylalanine and tyrosine do.*

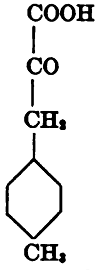
(3) *Para-methylphenylalanine and para-methoxy-phenylalanine, together with the corresponding ketonic acids, para-methyl-phenylpyruvic acid and para-methoxyphenylpyruvic acid, all of them substances incapable of yielding para-quinonoid derivatives. yield aceto-acetic acid and acetone when perfused through the surviving liver of the dog.*



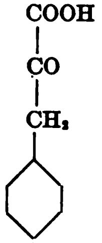
Para-methyl-
phenylalanine



Para-methoxy-
phenylalanine



Para-methyl-
phenylpyruvic
Acid



Para-methoxy-
phenylpyruvic
Acid

It is therefore clear that the series of reactions resulting in the production of aceto-acetic acid from tyrosine does not necessarily depend upon the prior formation of either a quinonoid intermediary substance or of homogentisic acid.

(4) The fact that substitution of the hydrogen atom in the para-position in phenylalanine by a methyl or methoxyl group does not interfere with its undergoing catabolism in the normal organism along similar lines to those of phenylalanine itself, is additional evidence supporting the view that phenylalanine is not necessarily converted into tyrosine in the course of its breakdown in the animal body.

(5) The most convincing evidence that the type of change which

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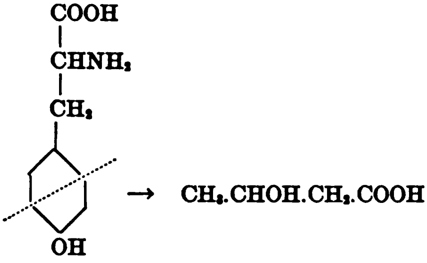
[1] The experiments with para-methoxy-phenylalanine have already been reported. This *Journal,* viii, p. 11, 1910.

in the alcaptonuric results in the conversion of tyrosine into homogentisic acid is *not* the only route for the aromatic amino-acids to follow is found in the fact that if the previously mentioned synthetic amino-acids, para-methylphenylalanine and para-methoxy-phenylalanine are fed to an alcaptonuric, they are, within reasonable limits, completely oxidized. It therefore follows that even the alcaptonuric is provided with a mechanism for the oxidation of the aromatic nucleus of amino-acids, provided that their conversion into homogentisic acid is prevented by suitable substitution in the para-position. (Cf. the following paper.)

Assuming that the above raised objections to Neubauer's formulation of the normal course of catabolism of phenylalanine and tyrosine are sustained, the next step is obviously to try and substitute some other scheme which is more in harmony with the facts. The question at present practically assumes the form of trying to picture the conversion of tyrosine into aceto-acetic acid (since this change has been demonstrated by Embden and others), without the intermediary formation of homogentisic acid. In considering the formula for tyrosine the possibility presents itself that the carbon atoms adjacent to the hydroxyl group in the para-position might in the course of some molecular rearrangement be converted into β-hydroxybutyric acid, which would in turn be oxidized to aceto-acetic acid and acetone.



$\rightarrow$ $CH_3.CHOH.CH_2.COOH$

This view is, however, untenable, in view of the formation of aceto-acetic acid and acetone from amino-acids in which no hydroxyl group in the para-position can be introduced, owing to that position being already substituted. We are thus led to consider the portion of the tyrosine molecular to which the side-chain is attached. It is certain that the carboxyl group present in the tyrosine or phenylalanine molecule is not identical with that which appears in the aceto-acetic acid molecule. Neubauer's results indicate most clearly the intermediary formation of α-ketonic acids followed by their oxidation with formation of an acid with one fewer carbon atom. When this is taken into account, along with the fact that aceto-acetic acid and acetone formation from phenylalanine derivatives is not inhibited by substitution in the para-position, and furthermore the fact that benzene itself may be oxidized in the animal organism to muconic acid,[1] it appears practically certain that *phenylalanine or tyrosine yields aceto-acetic acid in such a way that the carbon atom of the carboxyl group and that in the α-position in aceto-acetic acid are derived from the α and β carbon atoms of the phenylalanine side chain while the remaining β and γ carbon atoms of the aceto-acetic acid molecule are derived from two adjacent carbon atoms in the benzene nucleus.* The fact that phenylacetic acid does not yield aceto-acetic acid when perfused through a surviving liver may possibly be taken as indicating that disruption of the aromatic ring occurs prior to the oxidation of the α-ketonic acid in the α-position. At present we have not sufficient evidence to completely fill in the whole of the intermediate steps in the series of reactions but the scheme on the following page indicates the particular carbon atoms which it is believed are concerned in the formation of aceto-acetic acid.

This view of the formation of aceto-acetic acid furnishes an explanation of the very different behavior of phenylalanine and of phenlserine, $C_6H_5.CHOH.CHNH_2.COOH$, in the animal body.[2] The latter has been shown by one of us to yield hippuric acid when administered to cats and this failure to undergo complete oxidation in the body may fairly be ascribed to the impossiblilty of the formation of aceto-acetic acid from this substance owing to the presence of the hydroxyl group in the position adjacent to the benzene nucleus. The behavior of phenylserine in the body may be taken as additional evidence of the correctness of the suggestion put forward as to the mode of catabolism of phenylalanine and of tyrosine.

The fact that phenylamino-acetic acid and phenyl-α-amino-butyric acid in contrast to phenyl-α-aminopropionic acid (phenylalanine) do not undergo complete oxidation in the animal body, the benzene nucleus in both cases remaining intact is readily explained on the basis of the foregoing hypothesis as to the mode of catabolism of phenylalanine and tyrosine. Neither phenylamino-acetic acid nor phenyl-α-aminobutyric acid can for structural reasons yield aceto-acetic acid in the same way as phenylalanine and tyrosine do, if the suggested mechanism of the formation of the latter substance is correct.

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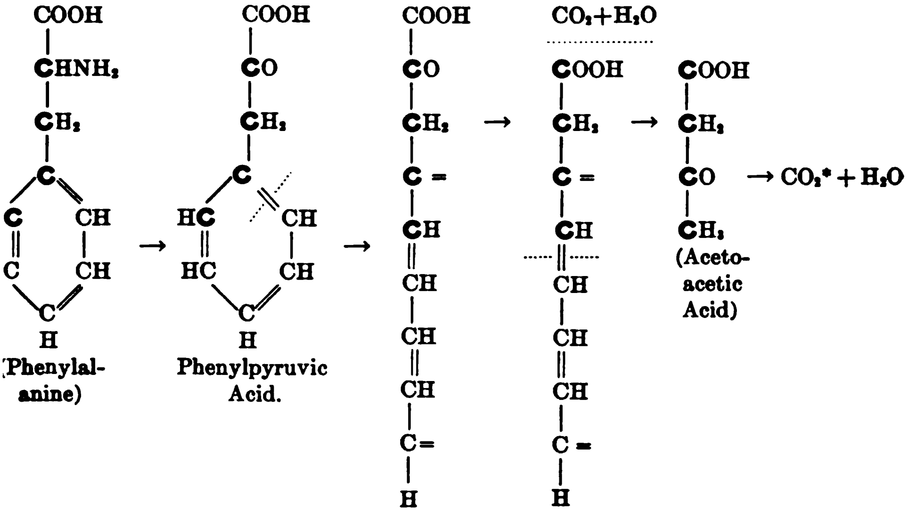
[1] Jaffe: *Zeitschr. f. physiol. Chem.*, lxii, p. 58.

[2] This *Journal*, vi, p. 238, 1909.



(Phenylpyruvic Acid written in open chain form.)

To sum up, it appears probable from a consideration of all the available evidence that of the nine carbon atoms in phenylalanine and tyrosine that present in the form of a carboxyl group is liberated in the form of carbon dioxide. Of the eight remaining carbon atoms, the four indicated in the accompanying diagram by heavier type form the carbon chain of an aceto-acetic acid molecule. It is possible that the remaining four carbon atoms,

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[*] We have reason to believe that formic and probably acetic acid are intermediate products in the catabolism of aceto-acetic acid. The experiments bearing on this question will be published shortly.

those occupying positions 2, 3, 4 and 5 in the aromatic nucleus, may undergo rearrangement with formation of a second molecule of aceto-acetic acid. At present there is no direct evidence bearing upon this latter point.

EXPERIMENTAL.

Liver Perfusion Experiments. For the following experiments we made use of a very simple arrangement for the perfusion of the livers of dogs through the portal vein.

The animal was anesthetised with ether, morphine being avoided as apparently it had an injurious effect upon aceto-acetic acid formation by the liver. Artificial respiration was employed. The portal vein was then exposed for a short distance close to its entrance to the liver and two loose ligatures placed around the vein. Care was taken to ligature any tributaries entering the vein in close proximity to the liver. A third loose ligature was placed around the vena cava above the entrance of the renal veins. The animal was then completely bled from a cannula previously inserted in the carotid or femoral artery. The blood was whipped and added to the perfusion fluid which had been previously prepared. As soon as the bleeding was practically complete the ligature around the vena cava was tied, also the ligature around the portal vein, farthest removed from the liver. A large, long glass cannula was then inserted in the portal vein and connected by a rubber tube to a syphon placed in the flask containing the warmed perfusion fluid. Another cannula was tied in the vena cava between the heart and diaphragm. A rubber tube attached to the cannula carried away the perfusion fluid from the liver and delivered it into a large flask which was kept at a temperature of about 38 degrees. Under favorable conditions perfusion was commenced in less than five minutes after the death of the animal. The first portions of blood coming from the liver were separately collected, whipped and returned to the main supply. Oxygen was bubbled through the liquids in both the delivering and receiving flasks so that the blood was well oxygenated. The fluid after traversing the liver was returned to the delivery flask. The rate of per-

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[1] This *Journal*, vi, p. 238, 1909.

fusion ordinarily varies from a minimum of 70 cc. per minute to about 200 cc. per minute. The pressure varied from about 2.5–5 cm. of mercury and was kept as low as possible. There appear to be some advantages in perfusing the liver *in situ* as described, rather than to excise the organ. The animal's body was kept warm throughout the experiment. The perfusion ordinarily lasted one hour.

The perfusion fluid was composed of a mixture of defibrinated bullock's blood together with the dog's own blood, and a solution of the substance under investigation in 50–100 cc. of saline. The amino-acids were relatively sparingly soluble in cold salt solution so that the following method was employed for preparing them in solution. Two grams of the amino-acid, *e. g.*, para-methylphenylalanine or para-methoxyphenylalanine, were converted into the readily soluble hydrochlorides by warming with 50 cc. of $\frac{N}{5}$ hydrochloric acid. An amount of normal caustic soda solution (10 cc.) sufficient to exactly neutralize the hydrochloric acid was very rapidly added, followed immediately by 500 cc. of blood. In this way, the amino-acid has practically no opportunity of crystallizing out and is readily obtained in neutral solution dissolved in blood slightly diluted with salt solution. The ketonic acids investigated, para-methyl-phenylpyruvic acid and para-methoxy-phenylpyruvic acid were converted into their readily soluble neutral ammonium or sodium salts.

The aceto-acetic acid and acetone estimations were in most cases made by taking an aliquot part of the blood after perfusion, diluting freely with water acidified with phosphoric acid and distilling in capacious flasks after making liberal additions of paraffin wax to control the foaming. In one or two cases the blood was treated with Schmidt's mercuric chloride reagent before distillation but the results were similar in either case. The acetone present in the distillates was estimated by means of iodine solution in the usual way.

The principal results of the experiments are recorded in the following table.

NO.	WEIGHT OF DOG	SUBSTANCE PERFUSED	WEIGHT OF SUBSTANCE	TIME OF PERFUSION	WEIGHT OF ACETO-ACETIC ACID FORMED
	Kilo		*grams*	*minutes*	*milligrams*
I	30	Blank	0	60	26.2
II	30	Blank	0	60	28
III	23	Blank	0	60	32.5
IV	28	Phenylalanine	2.0	60	166
V	31	Para-methyl-phenylalanine	2.0	60	84.2
VI	14	Para-methyl-phenylalanine	2.0	85	78.4
VII	32	Para-methyl-phenylalanine	2.0	60	153
VIII	15	Para-methoxy-phenylalanine	2.0	80	186
IX	15	Para-methoxy-phenylalanine	1.5	60	98
X	22	Para-methyl-phenylpyruvic Acid	2.0	60	62.5
XI	30	Para-methyl-phenylpyruvic Acid	2.0	60	116
XII	35	Para-methoxy-phenylpyruvic Acid	2.0	75	119

The results of the experiments require little or no comment. It will be seen that definite positive indications of the formation of aceto-acetic acid, were obtained when each of the four para-substituted substances: *p*-methyl-phenylalanine, *p*-methoxy-phenylalanine, *p*-methyl-phenylpyruvic acid, *p*-methoxy-phenylpyruvic acid, was added to the blood used for perfusing the liver. An experiment confirmatory of Embden's results with phenylalanine is also included in the table.

The details of the preparation of the substances used for perfusion are given in the following pages.

Para-methyl-phenylalanine (*Para-tolylalanine*). This amino-acid was prepared from para-methylbenzaldehyde by a method similar to Erlenmeyer and Halsey's synthesis of phenylalanine. The details of the preparation are given in the following paper. The amino-acid crystallizes from water in prisms melting at 277–279.°

Para-methoxy-phenylalanine (*Tyrosine methyl ether*). The synthesis of this amino-acid has already been described.[1] The last step in the preparation involving the hydrolysis of the benzoyl derivative of the amino-acid is apt to result in the formation of a small amount of tyrosine as the result of the action of the hydrochloric acid upon the methoxyl group. It is important therefore not to use too concentrated hydrochloric acid for the hydrolysis. A specimen of para-methylphenylalanine which gave a distinct Millon reaction due to contamination with a trace of tyrosine was purified as follows. The amino-acid was dissolved in eight parts cold dilute nitric acid, sp. gr., 1.15 and allowed to stand over night. A small separation of nitro-tyrosine occurred while the methoxyphenylalanine was not nitrated under these conditions. The filtrate was neutralized with ammonia, concentrated in the water-bath and the amino-acid allowed to crystallize out. It was then washed with a little cold water and recrystallized from boiling water. The resulting methoxy-phenylalanine was perfectly free from tyrosine and gave no reaction with Millon's reagent.

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[1] This *Journal*, viii, p. 17, 1910.

Para-methylphenylpyruvic acid. This acid has apparently not been previously described. It was prepared by Plöchl's method by heating the product of the condensation of para-methylbenzaldehyde and hippuric acid with caustic soda solution. The condensation product was obtained by heating on the water-bath for half an hour a mixture of para-methylbenzaldehyde (1 mol.), powdered hippuric acid (1 mol.), powdered fused sodium acetate (1 mol.) and acetic anhydride (2 mols.) The yellow "azlactone" readily separates out and is purified by washing with water and recrystallizing from alcohol. The substance melts at 141–142°. (Cf. following paper.) On boiling the "azlactone" (15 gms.) with fifteen times its weight of 40 per cent caustic soda solution for an hour, ammonia is freely evolved. On cooling and acidifying with a mixture of equal parts of crushed ice and concentrated hydrochloric acid a precipitate of impure benzoic acid is at once obtained. This precipitate is filtered off after a few minutes and the filtrate placed in a cool place. The crude ketonic acid slowly separates out of solution on long standing in a cool place and is purified by recrystallization from water containing a little alcohol. The substance crystallizes in prismatic needles, m. p., 178–180,° is readily soluble in alcohol and in ether. Its alcoholic solution gives a blue-green coloration on addition of ferric chloride.

Analysis: 0.1197 gm. gave 0.2981 gm. CO_2 and 0.0575 gm. H_2O.

	Found:	Calculated for $C_{10}H_{10}O$:
Carbon	67.9	67.4
Hydrogen	5.4	5.6

Para-methoxy-phenylpyruvic-acid. This acid has apparently not been previously described. It was prepared in precisely the same manner as the preceding ketonic acid by heating the condensation product of anisaldehyde and hippuric acid with strong caustic soda. This "azlactone" has already been described.[1] The ketonic acid slowly separates out from solution in the form of opaque needles. The acid is purified by repeated recrystallization from boiling water and melts at 190–192 degrees. It is very readily soluble in alcohol and ether, moderately soluble in boiling water, sparingly soluble in cold water. The alcoholic solution of the substance gives a deep blue coloration on addition of ferric chloride.

Analysis: 0.1275 gm. gave 0.02910 gm. CO_2 and 0.0574 gm. H_2O

	Found:	Calculated for $C_{10}H_{10}O_4$:
Carbon	62.2	61.9
Hydrogen	5.0	5.1

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[1] This *Journal*, viii, p. 19, 1910.

THE CHEMICAL NATURE OF ALCAPTONURIA.

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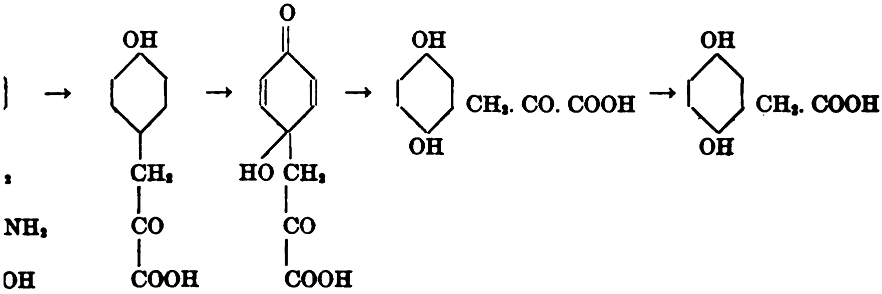
(Received for publication, February 16, 1911.)

The fundamental facts concerning alcaptonuria were established by Wolkow and Bauman[1] who determined the constitution of the peculiar acid found in the urines of alcaptonuric subjects and indicated its origin from tyrosine. Later Falta and Langstein[2] showed that the homogentisic acid might also be derived from phenylalanine.

The nature of the chemical reactions involved in this remarkable chemical transformation have long been a subject of speculation. Recently Neubauer[3] has put forward the following representation, which harmonizes admirably with all the known facts:



osine p-Hydroxyphenylpyruvic Acid p-Hydroxyphenylpyruvic Acid 2.5-Di-hydroxyphenylpyruvic Acid Homogentisic Acid

It will be seen that in this scheme the peculiar rearrangement of the relative position of the hydroxyl group and the side-chain present in tyrosine and homogentisic acid is accounted for by the assumption of the formation of an intermediary substance of quinonoid structure. There are many analogies in pure chemistry which make this assumption most probable.[1] Neubauer, Embden, Garrod and most other writers[2] regard this formation of homogentisic acid as a normal process in no way peculiar to the alcaptonuric. The abnormality in alcaptonuria, according to this view, consists in the failure to effect the oxidation of homogentisic acid. Embden has shown that homogentisic acid when perfused through a normal surviving liver yields aceto-acetic acid and acetone. The assumption is made therefore that the normal organism forms homogentisic acid but converts it as fast as it is formed into aceto-acetic acid or allied substances while in the alcaptonuric organism the homogentisic acid is excreted unchanged owing to failure to effect its conversion into aceto-acetic acid.

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[1] *Zeitschr. f. physiol. Chem.*, xv, p. 228, 1891.

[2] *Ibid.*, lxxviii, p. 161, 1903; *Deutsches Archiv f. klin. Med.*, lxxxi, p. 250, 1904.

[3] *Deutsches Archiv f. klin. Med.*, xcv, p. 254, 1909.

In the preceding paper reasons have been advanced for the belief that homogentisic acid is not an intermediate substance in the normal catabolism of tyrosine or phenylalanine. The evidence for this belief is somewhat intricate and will not be reproduced again in this paper. Assuming that this belief is correct, the inference must be drawn that alcaptonuria represents a condition in which there is not only an abnormal failure to deal with homogentisic acid when formed but also an abnormal production of homogentisic acid. It was considered probable that evidence as to the accuracy of these views might be obtained by the following type of experiment: From what has been mentioned before it appears practically certain that the formation of homogentisic acid from tyrosine is preceded by the formation of an intermediary substance of quinonoid structure. Now it is possible to prepare substituted derivatives of phenylalanine and of tyrosine in which the formation of a quinonoid intermediary substance is impossible and which therefore presumably could not yield homogentisic acid derivatives when fed to the alcaptonuric. Two substances of this type, para-methylphenylalanine and para-methoxyphenylalanine, have been prepared and their behavior in the normal and alcaptonuric organisms has been studied. It has been found that in the case of both the alcaptonuric and normal organism the substances are oxidized completely within reasonable limits. No excretion of homogentisic acid or derivatives of it followed the administration of these substances. *It therefore follows that the alcaptonuric has not lost the power to effect the catabolism of simple derivatives of phenylalanine or tyrosine provided their structure is such that formation of substances of the type of homogentisic acid is precluded. The inference certainly appears probable that alcaptonuria represents a condition in which the formation of homogentisic acid is abnormal as well as the failure to effect its catabolism when formed.*

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[1] Cf. the previous paper and This *Journal*, viii, p. 13, 1910.

[2] Cf. Garrod "*Inborn Errors of Metabolism*," Oxford Med. Publications, 1909.

Information as to the mode of break down of the para-methylphenylalanine and para-methoxyphenylalanine in the normal organism was obtained by observing the formation of aceto-acetic acid when they were perfused through a surviving dog liver. (Cf. previous paper.) It is probable that the same mechanism effects their catabolism in the alcaptonuric.

When large quantities (5 grams) of inactive para-methylphenylalanine were fed to an alcaptonuric a small amount of a crystalline substance was separated from the urine which proved to be the dextro-rotatory acetyl derivative of para-methylphenylalanine. The formation of this substance is of interest since Knoop[1] observed a similar acetylation of an amino-acid in the animal body, in the case of phenyl-α-aminobutyric acid. Neubauer[2] has observed the occurrence of acetylation when phenyl-α-amino acetic acid was perfused through a surviving liver and also when the same amino-acid is subjected to the action of actively fermenting yeast.[3] The mechanism of this peculiar formation of acetyl derivatives of amino-acids is quite unknown and would be an interesting subject for investigation.

When relatively large quantities of para-methylphenylalanine (2.5 grams) were fed to cats, a small quantity (8 per cent) of para-methylphenylacetic acid was found in the urine together with a little para-methyl-α-uramido-phenylpropionic acid. The formation of the para-methylphenylacetic acid may in part be ascribed to putrefactive action in the intestine. The formation of the uramido-acid is entirely similar to the excretion of α-uramido-phenylpropionic acid following the administration of large quantities of phenylalanine.[1] The behavior of para-methoxyphenylalanine when fed in large quantities to cats has already been reported.[2] The amino-acid even in very large quantities is oxidized as readily as tyrosine, although very small amounts of para-methoxyphenylacetic acid may be detected in the urine.

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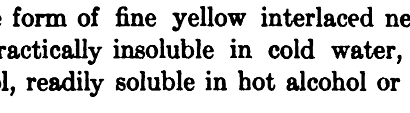
[1] *Zeitschr. f. physiol. Chem.*, lxvii, p. 497, 1910.

[2] *Ibid*, lxx, p. 1, 1910.

[3] *Ibid.*, p. 326, 1911.

EXPERIMENTAL.

Synthesis of Para-methyphenylalanine. This substance was obtained from para-tolylaldehyde by a series of reactions similar to those employed by Erlenmeyer and Halsey in their synthesis ot phenylalanine. Para-tolylaldehyde (1 mol.), powdered hippuric acid (1 mol.), fused sodium acetate (1 mol.), acetic anhydride (2 mols.) were heated on the water-bath for half an hour. A mass of yellow crystals of the condensation product separates and is filtered off, washed with water and purified by recrystallization from alcohol. The yield is about eighty per cent of the theoretical amount. The "azlactone" crystallised from alcohol or from ethylacetate in the form of fine yellow interlaced needles, m. p. 141–142°. It is practically insoluble in cold water, sparingly soluble in cold alcohol, readily soluble in hot alcohol or ethylacetate.



Analysis: 0.1720 gm. gave 0.4881 gm. CO_2 and 0.0789 gm. H_2O.
0.2725 gm. gave NH_3 = 0.0146 gm. N.

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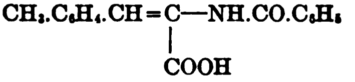
[1] Dakin: This *Journal*, vi, p. 235, 1910.

[2] This *Journal*, viii, p. 21, 1910.

	Found:	Calculated for $C_{17}H_{13}O_2N$:
Carbon	77.4	77.6
Hydrogen	5.1	4.9
Nitrogen	5.3	5.3

On dissolving the finely divided "azlactone" in hot 5 per cent. caustic soda solution and subsequently precipitating with hydrochloric acid, an excellent yield of α-benzoylamino-para-methylcinnamic acid



$$CH_3.C_6H_4.CH=\underset{\substack{|\\ COOH}}{C}—NH.CO.C_6H_5$$

was obtained. The substance is very sparingly soluble in cold water but readily soluble in strong alcohol. It crystallizes from dilute alcohol in long colorless needles arranged in the form of rosettes, melting with decomposition at 226–227°.

ANALYSIS: 0.1572 gm. gave 0.4176 gm. CO_2 and 0.0779 gm. H_2O.
0.1751 gm. gave NH_3 = 0.0086 gm. N.

	Found:	Calculated for $C_{17}H_{15}O_3N$:
Carbon	72.3	72.6
Hydrogen	5.5	5.3
Nitrogen	4.9	5.0

The α-benzoylamino-para-methylcinnamic acid (50 grams) was reduced by suspending it in half a liter of water and adding fifteen times its weight of 2.5 per cent. sodium amalgam fairly rapidly. The mixture is vigorously shaken and maintained at a temperature of about 40–50° for three hours. The alkaline solution was then acidified with hydrochloric acid. A gummy mass of benzoyl-para-methyl-phenylalanine separated out which slowly hardened on standing giving a semi-crystalline mass. The substance may be purified by crystallization from acetic acid but this entails considerable loss. It was found more economical to at once hydrolyze the crude benzoyl derivative by boiling with 1500 cc. of 20 per cent. hydrochloric acid for ten hours. On cooling, benzoic acid separated out and was removed by filtration. The filtrate was then evaporated practically to dryness *in vacuo*. The residue, consisting mainly of the hydrochloride of the desired methylphenylalanine, was dissolved in water and filtered. On adding ammonia gradually to the acid solution a small amount of pigmented impurity is precipitated. On complete neutralization the amino-acid readily crystallised out of solution and further quantities were obtained by concentrating the solution. The crude para-methylphenylalanine was purified by washing with a little cold water, followed by hot alcohol and finally ether. It was then recrystallized by dissolving in normal hydrochloric acid and adding an equivalent amount of ammonia to the clear solution. Para-methyl-phenylalanine is insoluble in alcohol, sparingly soluble in cold water and only moderately soluble in hot water. It crystallizes in colorless prisms melting with decomposition at 277–279°. It yields a finely crystalline hydrochloride sparingly soluble in concentrated hydrochloric acid, readily soluble in water.

Analysis: 0.1217 gm. gave 0.3352 gm. CO_2 and 0.0773 gm. H_2O.

	Found:	Calculated for $C_{10}H_{13}O_2N$:
Carbon..........................	66.9	67.0
Hydrogen......................	7.5	7.3

Para-methoxyphenylalanine. The synthesis of this amino-acid has already been described.[1]

Fate of Para-methylphenylalanine and Para-methoxyphenylalanine in the Alcaptonuric and Normal Organisms:

The amino-acids were mixed with food, usually cereal, and consumed in three portions at the close of each meal. No symptoms were noted that could be ascribed to the substances although one administration of methylphenylalanine was followed by moderately severe headache. The alcaptonuric subject was a typical case in all respects. Dr. Helen Baldwin, to whom I am indebted for much practical help, will report the case, so that further details are at present unnecessary. Before and during the administration of amino-acids the diet was maintained practically constant in quality although the quantity consumed varied somewhat.

The following table indicates the absence of any increase of homogentisic acid excretion following the administration of para-methylphenylalanine and para-methoxyphenylalanine to the alcaptonuric. The homogentisic acid was estimated according to Garrod and Hartley's[2] modification of Baumann's[3] method.

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[1] This *Journal*, viii, p. 18, 1910.

[2] *Journ. of Physiol.*, xxxiii, p. 206, 1905.

[3] *Zeitschr. f. physiol. Chem.*, xvi, p. 268, 1892.

VOLUME OF URINE	NITROGEN PER 24 HOURS	HOMOGENTISIC ACID PER 24 HOURS	RATIO OF NITROGEN TO HOMOGENTISIC ACID	
cc.	*Grams.*	*Grams.*		
2900	27.3	12.9	47	
1555	17.7	8.8	50	
1620	18.0	7.1	40	(5.0 gm. para-methylphenylalanine.)
1960	17.7	8.6	49	
1170	16.7	6.9	40	
2010	24.1	11.0	46	
2100	25.0	12.2	49	(5.0 gm. para-methoxyphenylalanine,
2080	19.6	9.4	48	

Apart from the estimation of homogentisic acid and total nitrogen in the urines, examinations were made to try and isolate any products of catabolism of the amino-acids and also to detect any unchanged amino-acid. The absence of any significant amount of unchanged methyl and methoxy-phenylalanines was shown as follows: measured portions of the urine before and after the feeding of the amino-acids were acidified with sulphuric acid and thoroughly extracted with ether to remove homogentisic acid. Excess of baryta solution was next added and the ammonia removed by an air blast. The solutions were then filtered, made up to definite volume, neutralized and then titrated with alkali in the presence of formaldehyde in the usual fashion. No increase in the "amino-acid" nitrogen of the urine could be detected.

The remainder of the urines secreted during the two days following the administration of para-methylphenylalanine was acidified with sulphuric acid and extracted with ether for 48 hours in a continuous extractor. The ether extract was distilled to complete dryness, warmed with 40 cc. of water and allowed to stand in a cold place for 24 hours. Only a trifling separation of oily matter occurred. Its amount was not greater than was obtained from an equal volume of urine collected at other times. Its acidity was equal to 3.0 cc. decinormal sodium hydrate. No large amount of sparingly soluble methylphenylacetic acid could therefore have been present. The aqueous filtrate on shaking vigorously with chloroform deposited a considerable quantity of crystals which slowly dissolved on shaking with larger volumes of chloroform. The chloroform extract on partial evaporation deposited the substance in the form of colorless needles. The total yield of crystals was 1.1 gram. The substance which proved to be dextro-rotatory acetyl-para-methylphenylalanine was readily soluble in alkali and also in strong hydrochloric acid. It dissolves readily in boiling water but is sparingly soluble in chloroform or benzene. It is conveniently crystallized from chloroform and melts at 170–171°. Its aqueous solution reacts acid to litmus.

Analysis: 0.1010 gm. gave 0.2993 gm. CO_2 and 0.0636 gm. H_2O.
. 0.0810 gm. gave $NH_3 = 0.0051$ gm. N.

	Found:	Calculated for $C_{12}H_{15}O_3N$:
Carbon.....	64.9	65.2
Hydrogen......................	7.0	6.8
Nitrogen........................	6.3	6.3

A molecular weight determination made by titrating an aqueous solution of the substance with standard alkali gave 228, compared with a calculated molecular weight of 221.

A determination of the acetyl group was made as follows: 0.3 gram substance was boiled for half an hour with 150 cc. of 3 per cent. sulphuric acid. The solution was then distilled to about 30 cc., water added and the distillation repeated four times. The volatile acid in the distillate required 12.8 cc. of decinormal alkali to neutralize it, as against 13.1 cc. required by theory. That the volatile acid was acetic acid was proved by converting it into the barium salt in the usual way.

Analysis: 0.1584 gm. salt gave 0.1203 gm. $BaCO_3$.

	Found:	Calculated for $C_4H_6O_4Ba$:
Barium..........................	53.3	53.8

The acetyl-p-methylphenylalanine was strongly dextro-rotatory although the observed value for $[\alpha]_D^{20°}$ can only be regarded as approximate on account of the small amount of material available:

$$c = 1.126;\ l = 1;\ \alpha = +0.39°$$
$$[\alpha]_D^{20°} = +34.6°$$

The aqueous solution from which the acetyl compound had been extracted with chloroform on further examination yielded no other substance than homogentisic acid together with traces of hippuric acid.

In a second experiment in which 2.5 grams of methylphenlalanine was fed to the alcaptonuric no acetyl or other derivative could be detected in the urine.

The urine secreted during the period following the feeding of 5.0 grams of para-methoxyphenylalanine was examined as in the case of methylphenylalanine. No indications of unchanged amino-acid were obtained, neither was any significant amount of methoxyphenylacetic acid present. Apparently practically the whole of the substance had undergone complete oxidation.

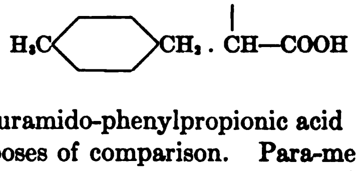
When quantities of three grams of *p*-methylphenylalanine and five grams of *p*-methoxyphenylalanine were consumed by a normal individual no crystalline derivatives of these amino-acids could be separated from the urines.

On feeding 2.5 grams of *p*-methylphenylalanine to cats and analysing the urine precisely as was done in the case of the similar experiments already reported, in the case of *p*-methoxyphenylalanine, small quantities of two derivatives were obtained. The first proved to be *p*-methylphenylacetic acid which separated from solution on dissolving the ether extract in warm water and allowing the solution to stand. It was purified by successive conversion into the ammonium and silver salts. The yield of *p*-methylphenylacetic acid in two similar experiments was about 0.2 gram.

ANALYSIS: 0.1877 gm. Ag. salt gave 0.0780 gm. Ag.

	Found:	Calculated for $C_9H_9O_2Ag$:
Silver........................	41.6	42.0

The second derivative proved to be para-methyl-uramidophenylpropionic acid. It separated from solution in very small quantity on concentrating the aqueous solution of the ether extract after the separation of the methyl-phenylacetic acid. The substance was sparingly soluble in water and melted at 195–196° with effervescence.



$$H_3C\langle\quad\rangle CH_2 . \underset{}{\overset{NH-CO-NH_2}{\overset{|}{CH}}}-COOH$$

Para-methyl-α-uramido-phenylpropionic acid was prepared synthetically for purposes of comparison. Para-methylphenylalanine (1.0 gm), potassium cyanate (1.0 gm.) and water (10 cc.) were mixed together and heated for one hour on the water-bath. The residue was dissolved in water and the solution acidified with hydrochloric acid. The uramido-acid at once separated from solution and was recrystallised from boiling water. The yield was about 70 per cent of the theoretical amount. The substance crystallized in small prisms melting with effervescence at 195–196° and was identical with the product separated from urine.

Analysis: 0.1518 gm. gave 0.3312 gm. CO_2 and 0.0890 gm. H_2O.

	Found:	Calculated for $C_{11}H_{14}O_3N_2$:
Carbon	59.5	59.4
Hydrogen	6.5	6.3

SUMMARY.

Evidence is adduced to show that the condition of alcaptonuria does *not* simply represent a failure to oxidise homogentisic acid.

The present investigations indicate that the formation of homogentisic acid from tyrosine or phenylalanine is probably abnormal. By feeding an alcaptonuric simple derivatives of phenylalanine and tyrosine (para-methylphenylalanine and para-methoxyphenylalanine) it was found that these amino-acids apparently underwent practically complete oxidation. It follows therefore that the alcaptonuric organism has not lost its ability to catabolize simple derivatives of phenylalanine and tyrosine provided their structure is such that formation of substances of the type of homogentisic acid is excluded.

It appears probable that alcaptonuria represents a condition in which there is an abnormal formation of homogentisic acid in addition to an abnormal failure to catabolize it when formed.

The behavior of para-methylphenylalanine and para-methoxyphenylalanine when fed to normal human beings and to cats was also investigated.

RESEARCHES ON PURINES.

ON 2-OXY-9-METHYLPURINE AND 2,8-DIOXY-9-METHYLPURINE.

THIRD PAPER.[1]

By CARL O. JOHNS.

(*Contributions from the Sheffield Laboratory of Yale University.*)

(Received for publication, February 25, 1911.)

Three of the six 2-oxymonomethylpurines required by theory have been described. These are 2-oxy-3-methylpurine[2] (I), 2-oxy-6-methylpurine[3] (II) and 2-oxy-7-methylpurine[4] (III).

The writer has now prepared 2-oxy-9-methylpurine (X).

It is well known that formic acid, acetic anhydride, urea and thiourea condense with orthodiaminopyrimidines to give purines. Apparently no one hitherto has tried to condense these reagents with orthodiaminopyrimidines in which a hydrogen atom of one or both amino groups has been substitued by an alkyl group. The writer finds that 2-oxy-5-amino-6-methylaminopyrimidine (XI) reacts even more readily with formic acid and urea than does the corresponding unsubstituted pyrimidine.

The following reactions led to the synthesis of 2-oxy-5-amino-6-methylaminopyrimidine. 2-Ethylmercapto-6-chlorpyrimidine[5] (VI) was heated at 100° C. with an aqueous solution of methylamine. The resulting 2-ethylmercapto-6-methylaminopyrimidine (IX) was boiled with concentrated hydrochloric acid to obtain 2-oxy-6-methylaminopyrimidine (VIII). This compound was dissolved in sulphuric acid and nitrated with fuming nitric acid giving 2-oxy-5-nitro-6-methylaminopyrimidine (VII). In all of these reactions the yields were almost quantitative. The most difficult step was the reduction of 2-oxy-5-nitro-6-methylaminopyrimidine. This was accomplished by the use of ferrous hydroxide and yields of 70–75 per cent of 2-oxy-5-amino-6-methylaminopyrimidine (XI) were obtained. This compound was converted to 2-oxy-9-methylpurine (X) by boiling with formic acid and evaporating to dryness at 130–140° C.

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[1] *Amer. Chem. Journ.*, xli, p. 58, 1909. *Ibid.*, xlv, p. 79, 1911.

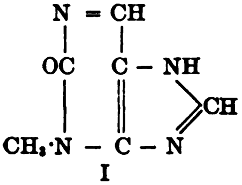
[2] Tafel and Weinschenk: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 3372, 1900.

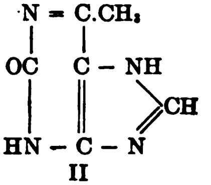
[3] Johns: *Amer. Chem. Journ.*, xli, p. 65, 1909.

[4] Emil Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxxi, p. 2554, 1898.

[5] Wheeler and Johnson: *Amer. Chem. Journ.*, xxix, p. 496, 1903.

Theoretically, five monomethyl-2,8-dioxypurines are possible. Two of these isomers have already been described, namely, 2,8-dioxy-3-methylpurine[1] (IV) and 2,8-dioxy-6-methylpurine[2] (V).


I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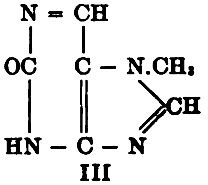
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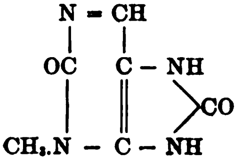
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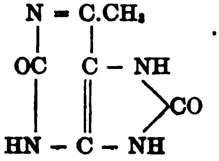
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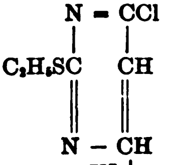
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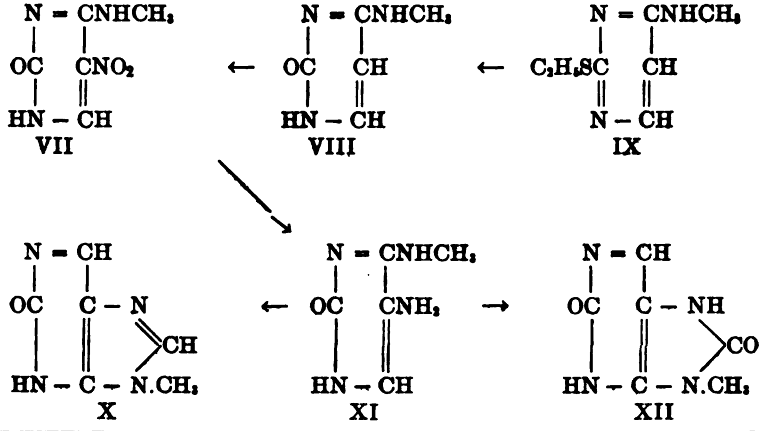
VII

VIII

IX

X

XI

XII

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[1] Fischer and Ach: *Ber. d. deutsch. Chem. Gesellsch.*, xxxii, p. 2736, 1899.

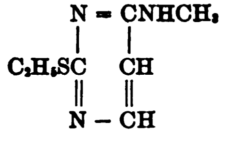
[2] Johns: *Amer. Chem. Journ.*, xli, p. 63, 1909.

When 2-oxy-5-amino-6-methylaminopyrimidine is heated with urea it gives 2,8-dioxy-9-methylpurine (XII).

These researches will be continued.

EXPERIMENTAL PART.

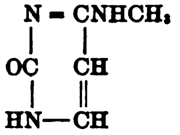
2-Ethylmercapto-6-methylaminopyrimidine.



Ten grams of 2-ethylmercapto-6-chlorpyrimidine[1] were mixed with 14 grams of 33 per cent aqueous methylamine and heated in a sealed tube at 100° C. for three hours. Before heating, the mixture was opaque but when the reaction was complete it gave a heavy transparent oil. The oil solidified to a crystalline mass at room temperature. The crystals were washed with water and dried over sulphuric acid. The yield was almost quantitative. The crystals obtained were very soluble in ether, benzene, alcohol, or 20 per cent hydrochloric acid. They were insoluble in water and were crystallized from very dilute alcohol. On cooling the solution slowly small stout prisms were obtained. These melted to a colorless oil at 55° C.

	Calculated for $C_7H_{11}SN_3$:	Found:
N	24.85	24.73

2-Oxy-6-methylaminopyrimidine.



Ten grams of 2-ethylmercapto-6-methylaminopyrimidine were dissolved in 100 cc. of concentrated hydrochloric acid and the solution was boiled for three hours after which it was evaporated to complete dryness. A white residue, which proved to be a hydrochloride, remained. This was taken up in 20 cc. of water and the solution was neutralized with ammonia. The free base separated as a white crystalline precipitate. It was very soluble in hot and moderately soluble in cold water. It crystallized from water in small stout prisms. These were washed with alcohol and dried at 100° C. They were not soluble in ether or benzene, but were moderately soluble in hot and difficultly soluble in cold alcohol. They began to darken at 250° C. and melted with decomposition at 270° C. The yield was quantitative.

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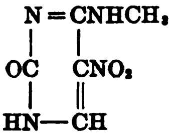
[1] Wheeler and Johnson: *Loc. cit.*

	Calculated for $C_5H_7ON_3$:	Found:
N	33.60	33.61

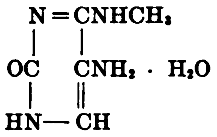
2-Oxy-5-nitro-6-methylaminopyrimidine.



Ten grams of 2-oxy-6-methylaminopyrimidine were dissolved in 20 cc. of cold concentrated sulphuric acid and 10 cc. of nitric acid (sp. gr. 1.5) were added gradually. Nitration took place at once without oxidation. The resulting mixture was poured into 100 cc. of cold water and the acids were neutralized with ammonia. This produced an immediate crystalline precipitate. This was insoluble in ether, benzene, or alcohol but dissolved in 20 per cent hydrochloric acid and dilute ammonia. It was difficultly soluble in hot water from which it crystallized in minute prisms that had the appearance of broken columns when viewed under the microscope. The yield was quantitative. The nitro compound blackened rapidly above 300° C.

	Calculated for $C_5H_6O_3N_4$:	Found:
N	32.94	32.75

2-Oxy-5-amino-6-methylaminopyrimidine.



$$\begin{array}{ll} N = & CNHCH_3 \\ | & | \\ OC & CNH_2 \cdot H_2O \\ | & \| \\ HN - & CH \end{array}$$

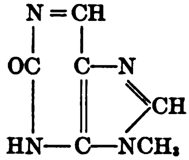
Twelve grams of 2-oxy-5-nitro-6-methylaminopyrimidine were dissolved in 150 cc. of concentrated ammonia which had been diluted with 100 cc. of water. This solution was mixed with a solution of 180 grams of crystallized ferrous sulphate in hot water. Reduction began at once and considerable heat was evolved. An aqueous solution of 210 grams of crystallized barium hydroxide was added to precipitate the sulphate. A slight excess of barium hydroxide was removed by adding ammonium carbonate. The reduction was allowed to proceed over night at room temperature. The pricipitate was filtered off by suction and washed with hot water. The filtrate was concentrated to about 50 cc. Well defined, slender prisms with square ends separated on cooling. The yield was 70-75 per cent of the calculated. The methyldiaminopyrimidine was very soluble in hot water, but it crystallized out almost completely on cooling the hot concentrated solution. The crystals thus obtained contained one molecule of water of crystallization that could be driven off at 110° C. The anhydrous substance did not dissolve in ether or benzene. It was moderately soluble in hot and slightly soluble in cold alcohol. It began to blacken at 210° C and decomposed slowly above that temperature, effervescing at about 225° C.

I. 1.260 gm. of substance lost 0.145 gm. of H_2O at 130° C.
II. 0.6934 gm. of substance lost .0804 gm. of H_2O at 130° C.

	Calculated for $C_5H_8ON_4 . H_2O$:	Found:	
H_2O	11.39	11.50	11.59
		I.	II.

	Calculated for $C_5H_8ON_4$:	Found:
N	40.00	39.74

2-Oxy-9-methylpurine.

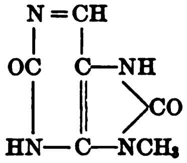


Five grams of anhydrous 2-oxy-5-amino-6-methylaminopyrimidine were heated in 20 cc. of 85 per cent formic acid at 100° C for one hour. The solution was then evaporated to dryness and finally heated for one hour at 130-140° C. to remove all of the formic acid. The yield of crude purine was quantitative. It was dissolved in very dilute ammonia and a little color was removed by boiling the solution with blood coal. When the solution was concentrated to 25 cc. and cooled slowly the purine separated as a mass of long slender prisms. These were soluble in less than five parts of boiling water and moderately soluble in cold water. They were sparingly soluble in hot and almost insoluble in cold alcohol. They did not dissolve in hot toluene. They were easily soluble in dilute mineral acids and bases. They began to turn brown at about 290° C. and decomposed with some effervescence at about 310° C. The air dried substance lost 8.3 per cent of water at 130° C., while the calculated loss for one molecule of water is 10.71 per cent. Determinations of water in other samples of the air dried substance gave results approximating 8.3 per cent. The calculated for three quarters molecule of water is 8.25 per cent. It is probable, however, that the compound crystallizes with one molecule of water, a part of which is lost on drying. This purine gave a brilliant murexide reaction.

0.1565 gm. of anhydrous substance gave 0.2773 gm. of CO_2 and .0579 gm. of H_2O.

	Calculated for $C_6H_6ON_4$:	Found:	
C	48.00	48.31	
H	4.00	4.11	
		I.	II.
N	37.33	37.32	37.12

2,8-Dioxy-9-methylpurine.



Five grams of 2-oxy-5-amino-6-methylaminopyrimidine and 5 grams of urea were pulverized together and the powder was heated in an oil bath at 150°-160° C. for one hour. Ammonia was evolved and the powder melted to a liquid which finally solidified to a hard cake. This was crushed and dissolved in hot water containing a little sodium hydroxide. A trace of insoluble matter was filtered off and the hot straw-colored solution was acidified with acetic acid. This produced a precipitate of very minute prisms. The yield was 85 per cent of the calculated quantity. This compound was easily soluble in dilute sodium hydroxide but sparingly soluble in dilute ammonia. It dissolved readily in mineral acids but the salts were dissociated by water. It was insoluble in acetic acid and the common organic solvents. It dissolved in less than 500 parts of boiling water. When heated with hydrochloric acid and potassium chlorate and evaporated to dryness the residue gave the murexide reaction when moistened with ammonia. It did not show any indications of melting at 315° C.

0.1825 gm. of substance gave 0.2910 gm. of CO_2 and 0.0591 gm. of H_2O

	Calculated for $C_6H_6O_2N_4$:	Found:	
C	43.37	43.47	
H	3.61	3.59	
		I.	II.
N	33.73	33.90	33.67

ON THE PHYSIOLOGICAL AGENTS WHICH ARE CONCERNED IN THE NUCLEIN FERMENTATION, WITH SPECIAL REFERENCE TO FOUR INDEPENDENT DESAMIDASES.

BY WALTER JONES.

(*From the Laboratory of Physiological Chemistry, Johns Hopkins University.*)

(Received for publication, March 21, 1911.)

In a recent communication[1] it was shown that when guanylic acid is submitted to the action of an aqueous extract of pig's pancreas, neither desamidization nor liberation of purine base occurs: and it was pointed out that this resistence of guanylic acid explains the curious conduct of the pancreas in self-digestion, viz. that no free guanine is formed in the digestion but that the base makes its appearance when the products of digestion are submitted to hydrolysis with boiling mineral acid. It was therefore concluded that guanylic acid is not changed by pig's pancreas. But all of the findings which were reported accord equally well with the assumption that, although guanylic acid yields no free guanine under the conditions stated, it nevertheless loses phosphoric acid with the formation of guanosine.

$$H_2PO_4.C_5H_8O_3.C_5H_2N_4O(NH_2)+H_2O=C_5H_9O_4.C_5H_2N_4O(NH_2)+H_3PO_4$$

The researches of Hahn and Geret,[2] Jwanoff[3] and others would indeed suggest by analogy that this view is correct. These writers have shown that under the influence of yeast, moulds and gland extracts, nucleic acids are decomposed with liberation of phosphoric acid; but in practically all of the experiments on the subject it has been observed that the liberation of phosphoric acid occurs in association with the liberation of purine bases; so that there is a degree of justification for assuming that a tissue extract which cannot liberate purine bases is exceptional and will be found also incapable of liberating phosphoric acid. But my conclusion that guanylic acid does not lose phosphoric acid through the action of pig's pancreas was partly founded upon some rather unsatisfactory polarimetric observations which indeed required corroboration, but which were sufficient to give me a somewhat decided opinion that the rotation of guanylic acid undergoes no change when the substance is digested with pig's pancreas.

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[1] Jones: *This Journal*, ix, p. 129.

[2] Hahn and Geret: *Zeitschr. f. Biol.*, xxxiv, p. 117.

[3] Jwanoff: *Zeitschr. f. physiol. Chem.*, xxxix, p. 31.

While my former publication was in press an article appeared by Levene and Medigreceanu[4] in which is described the action of the plasmata of dog's organs upon various substances related to nucleic acid. Among their experiments is one which deals with the conduct of guanylic acid toward dog's pancreas. The optical method was employed but owing to the cloudiness of the solutions the writers could not give a final opinion, although they were rather inclined to believe that guanylic acid does decompose, under the conditions stated, with the formation of guanosine.

My experience had taught me that the optical method is not well adapted to a decision of this question. I therefore proceeded with examinations for liberated phosphoric acid, and results were obtained which leave no room for doubt that by the action of pig's pancreas guanylic acid loses its phosphoric acid being thereby converted into guanosine. The results of my former paper should therefore be taken to mean that guanosine (not guanylic acid) is unaffected by this tissue extract[5]

The phosphoric acid set free from nucleic acid in tissue extracts is sharply precipitated by magnesia mixture and can be determined with great accuracy. This precipitate of magnesium ammonium phosphate is beautifully crystalline and doubtless very pure but in all experiments here reported the compound was converted into ammonium phosphomolybdate, this again into magnesium ammonium phosphate and the phosphorus finally weighed as magnesium pyrophosphate. It was thus found, that aqueous extracts of pig's liver, spleen and pancreas liberate phosphoric acid progressively as self-digestion proceeds and that both guanylic acid and thymus nucleic acid lose their phosphoric acid completely in the presence of aqueous extracts of these tissues at 40°. No injury to the ferment from trypsin was noticed for the pancreas was found the most active of the three glands.

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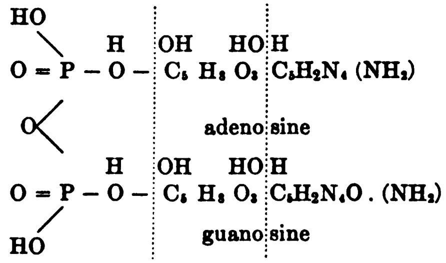
[4] Levene and Medigreceanu. This *Journal*, ix, p. 65.

[5] The statement also that guanylic acid does not prevent the precipitation of purine bases was based upon a misconception and is not correct. It is probably true for guanosine, but when a solution of guanylic acid and hypoxanthine is treated with ammoniacal silver nitrate no precipitate is formed.

The conduct of nucleic acids in the presence of extracts of these glands is therefore not difficult to study so far as concerns the purine groups. From the remarkable work of Levene and Jacobs[6] upon the structure of nucleic acids it is evident that when phosphoric acid is removed from such a compound by the action of gland extracts there must arise two nucleosides and they will be as surely present as though the isolated compounds themselves had been added.



Nucleic acid represented as a di-nucleotide.[7]

One of these nucleosides (guanosine) is composed of d-ribose and guanine, and upon acid hydrolysis gives guanine, directly precipitable with copper and silver salts. The other nucleoside (adenosine) is composed of d-ribose and adenine, and upon acid hydrolysis gives directly precipitable adenine.

By chemical methods, Levene and Jacobs have been able to transform these two nucleosides which are amido-derivatives into two nucleosides (xanthosine and inosine) which are the corresponding oxy-compounds. Thus

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[6] *Berichte d. d. chem. Ges.*, xxxxi, xxxxii, xxxxiii.

[7] Levene and Jacobs: *ibid.*, xxxxii, p. 2703.

$$\underset{\text{guanosine}}{C_5H_9O_4 \,.\, C_5H_2N_4O\ (NH_2)} + H_2O = \underset{\text{xanthosine}}{C_5H_9O_4 \,.\, C_5H_2N_4O\ (OH)} + NH_3$$

$$\underset{\text{adenosine}}{C_5H_9O_4 \,.\, C_5H_2N_4\ (NH_2)} + H_2O = \underset{\text{inosine}^{8}}{C_5H_9O_4 \,.\, C_5H_2N_4\ (OH)} + NH_3$$

The transformations are simple desamidizations perfectly analogous to those which occur when guanine and adenine are converted into xanthine and hypoxanthine respectively.[9]

It is easily possible to see which of these substances is present in a digested glandular extract by finding the purine base produced when the product is boiled with mineral acid. If one should find that hypoxanthine, for instance, is not directly precipitable from the product but is precipitated after hydrolysis he would be justified in assuming that the material contained inosine. If the substance had been produced from a nucleic acid (which *must* give adenosine initially) one would be equally justified in concluding that he is dealing with a desamidizing agent through whose influence adenosine has been converted into inosine.

It is thus proposed to show that pig's pancreas can effect the conversion of adenosine into inosine which is to say that the tissue contains adenosine-desamidase. But pig's pancreas exerts no similar action upon guanosine. (This is interesting since the pancreas promptly desamidizes free guanine). This conclusion follows from a study of the products formed by the action of the tissue extract on thymus nucleic acid. Phosphoric acid is split off (whose presence can be quantitatively determined) so that guanosine and adenosine must be formed; but the purine bases are not set free. By hydrolysis of the digested product guanine and *hypoxanthine* are set free and can be precipitated with silver or copper salts. Plainly the guanine comes from guanosine[10] while the hypoxanthine comes from inosine which was evidently produced from adenosine[11] during digestion. In the self-digestion of the pancreas it

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[8] The term "hypoxanthosine" would have more meaning but the substance was named before its constitution was understood.

[9] Levene and Jacobs: *ibid.*, xxxiii, p. 3150.

[10] This phenomenon was also observed when guanylic acid was employed instead of thymusn ucleic acid.

[11] Combined adenine can be shown in the earlier stages of the digestion at a time when all phosphoric acid has been split off.

was also found that the hypoxanthine is greatly increased when the digested product is boiled with mineral acid. The evidence here given for assuming the independence of these two nucleoside-desamidases is of the same nature as that which caused me to distinguish guanase and adenase from one another.[12] In each instance one of the two ferments exhibits its activity in the absence of the other so that the independent existence of two ferments must be assumed.

What has been stated of pig's pancreas is essentially true of ox pancreas. An extract of this gland liberates phosphoric acid from thymus nucleic acid with the necessary production of guanosine and adenosine. The former compound is not further altered since acid hydrolysis of the products produces guanine; but the adenosine is desamidized to inosine which gives rise to hypoxanthine when the product is boiled with acid.

The existence of adenosine-desamidase in the pancreas is thus shown but the analogous guanosine-desamidase has thus far only been intimated. Its existence however comes to light in a study of pig's liver. No examination of the action of this tissue extract upon thymus nucleic acid has been made, but the arrangement of ferments can be noted just as well by an examination of the products of self-digestion. It has been known for some time that the adenine group of nucleic acids is split off, desamidized and oxidized by the ferments of pig's liver and that the final product is xanthine. When the products of self digestion of the tissue are boiled with acid (as we would expect) there is no appearance of either adenine or hypoxanthine, but the *xanthine* is greatly increased without any increase of guanine. It therefore appears that the guanosine first formed from nucleic acid is desamidized, thereby giving rise to xanthosine which in turn yields xanthine upon hydrolysis. The formation of free guanine in the self-digestion of the liver as well as the formation of both free xanthine and free hypoxanthine in the self-digestion of the pancreas are matters which might have been expected. In the enzymic hydrolysis of nucleic acid there are two possibilities; phosphoric acid may be split off or purine bases may be set free. Advantage is taken of both opportunities and some free guanine and adenine are formed. The liver converts the adenine into xanthine (action of adenase and xanthooxidase[13]) while the guanine remains unchanged (guanase is not present in this tissue[14]) The pancreas converts both of the amidopurines into oxypurines but does not oxidize the hypoxanthine to xanthine (presence of guanase and adenase and absence of xanthooxidase).[15] The end products here stated are the ones actually found in the digestion of the tissues and to a slight extent also by the action of these tissues extracts upon thymus nucleic acid.

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[12] Jones and Winternitz: *Zeitschr. f. physiol. Chem.*, xliv, p. 1.
Jones. *ibid*, xlv, p. 83.

That guanosine-desamidase and guanase are different ferments is obvious: the one is present in pig's liver and the other is not. That adenosine-desamidase and adenase are not identical appears from the following considerations. I have found that a number of animal glandular extracts produce hypoxanthine upon self-digestion but are nevertheless incapable of converting adenine into hypoxanthine. This is true of dog's liver, rabbit's liver, a number of human organs, and practically all of the organs of the rat: in fact the occurrence of adenase is rare. In the self-digestion of these tissues the initial adenine is combined in the form of a nucleic acid; and if we assume that some ferment is present such as adenosine-desamidase which can desamidize an intermediate product but which cannot attack adenine, the conduct of these glands is explained.

The experiments described below were selected from a large amount of material obtained in a study of the action of glandular extracts upon thymus nucleic acid. These particular experiments are reported because they collectively show the factors which are concerned in the enzymic decomposition of nucleic acid. The general course of such a decomposition and the relation of the products to one another are graphically represented in the diagram.

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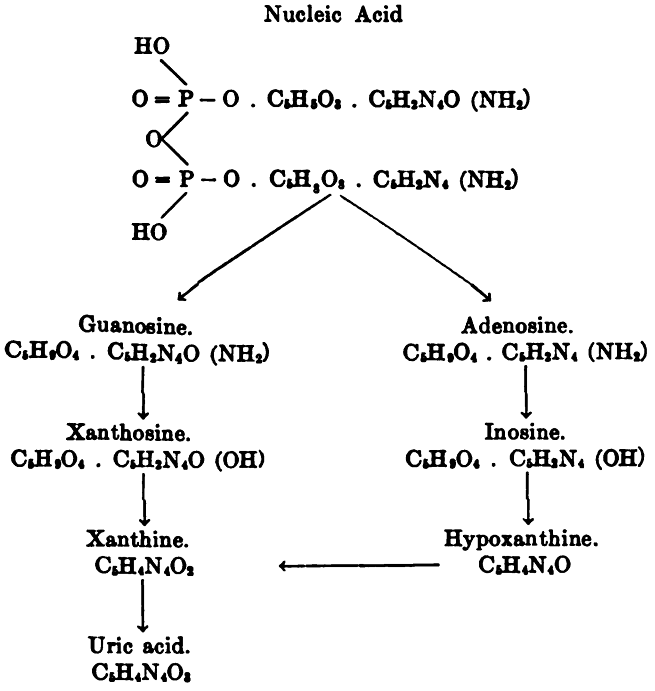
[13] Jones and Winternitz: *loc. cit.*

[14] *Ibid.*

[15] Jones and Partridge: *Zeitschr. f. physiol. Chem.*, xlii, p. 343.


Nucleic Acid
HO
O = P − O . $C_5H_8O_3$. $C_5H_3N_4O$ (NH_2)
O
O = P − O . $C_5H_8O_3$. $C_5H_3N_4$ (NH_2)
HO
Guanosine.
$C_5H_9O_4$. $C_5H_3N_4O$ (NH_2)
Adenosine.
$C_5H_9O_4$. $C_5H_3N_4$ (NH_2)
Xanthosine.
$C_5H_9O_4$. $C_5H_3N_4O$ (OH)
Inosine.
$C_5H_9O_4$. $C_5H_3N_4$ (OH)
Xanthine.
$C_5H_4N_4O_2$
Hypoxanthine.
$C_5H_4N_4O$
Uric acid.
$C_5H_4N_4O_3$

The diagram represents of course only one path that the fermentation may take. It is sure that in some cases the purine bases are directly liberated from the nucleic acid and possible that hydrolysis of the nucleosides precedes desamidization. In such cases two well known desamidases (guanase and adenase) come into play if they happen to be present in the tissue under consideration.

The action of ox spleen is very different from that of the tissues considered. Extracts of this gland completely liberate, desamidize, and oxidize both purine groups of a nucleic acid, the final product being xanthine.[16] In such a case it is not possible to say in what order the ferments act.

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[16] Uric acid, if a proper amount of air is admitted.
Schittenhelm. *Zeitschr. f. physiol. Chem.*, xlii, p. 251.

EXPERIMENTAL PART.

I. *Phosphoric Acid.*

In all determinations of phosphoric acid the following method was employed. Part of the product of digestion was boiled and filtered. After cooling, 200 cc. of the fluid were measured out, made alkaline with ammonia and treated with an excess of magnesia mixture. The precipitated magnesium ammonium phosphate was converted into ammonium phosphomolybdate, this again into ammonium magnesium phosphate which was heated and weighed as magnesium pyrophosphate and the corresponding amount of phosphoric acid calculated (as P_2O_5) which appears in the tables expressed in milligrams. In all auto-digestions the gland was mixed with three parts of water.

When a nucleic acid was used in connection with the gland extract, the latter was strained and divided into two parts. To one part the nucleic acid was added and the other was used as a control. The amount of nucleic acid in 200 cc. of the coagulated and filtered fluid was calculated as a fractional part of the entire substance used; and is designated in the tables as "nucleic acid involved." This method of procedure gives results which are much more accurate than those obtained after attempting to wash the coagula.

1. Self-digestion of pig's pancreas liver and spleen. Extract 1:3 without straining. 200 cc. of the product, after heating, and filtering, used in each determination. Phosphorus weighed as $Mg_2P_2O_7$ and expressed in milligrams as P_2O_5.

	PANCREAS	SPLEEN	LIVER
Fresh extract	37	35	Cloudy
17 hours at room temperature	126	109	122
24 hours longer at 40°	267	208	225
24 hours longer at 40°	264	228	247
A week at 40°	268	233	269

2. Action of strained extracts of pig's pancreas spleen and liver and ox pancreas on thymus nucleic acid. Digestion 7 days. 200 cc. of filtrate for each determination. Nucleic acid involved in each, 1 gm. equivalent to 200 mg. of P_2O_5.

	PIG'S PANCREAS	OX PANCREAS	PIG'S SPLEEN	PIG'S LIVER
Experiment	328	328	410	349
Control	138	141	220	172
From the thymus nucleic acid	190	187	190	177

3. The same with guanylic acid. Guanylic acid involved, 1 gm. Equivalent to 179 mg. P_2O_5. Digestion 7 days.

	PIG'S PANCREAS	OX PANCREAS	PIG'S SPLEEN	PIG'S LIVER
Experiment	309	303	390	346
Control	138	141	220	172
From guanylic acid	171	162	170	174

4. A portion of pig's pancreas extract (1:3 without straining) after digestion 7 days at 40° was heated and filtered. Ten portions of 200 cc. each were measured out and five treated in each of the two following ways.

a. The phosphoric acid was determined as described and weighed as magnesium pyrophophate.

b. The fluid was treated with magnesia mixture, the precipitate washed, filtered and dissolved in a slight excess of nitric acid. The solution was treated with a few drops of magnesia mixture and made alkaline with ammonia. The precipitated magnesium ammonium phosphate was beautifully crystalline, snow white and appeared homogeneous under the microscope. This was filtered off, allowed to dry for about 2 hours and weighed. The substance was then exposed to the air for a week and weighed again.

It will be observed that the results obtained by these two methods do not differ greatly and it is possible that those obtained by the shorter method are the correct ones.

	I	II	III	IV	V
P_2O_5 after full purification weighed as $Mg_2P_2O_7$	262	259	267	264	260
Weighed as $MgNH_4PO_4$ when apparently dry	280	271	274	278	279
Weighed as $MgNH_4PO_4$ after standing a week	280	269	271	277	276

II. *Purine Bases.*

The method employed for the isolation of the purine bases was described in a former paper. All extracts were made with one part of ground gland and three parts of water. In order to avoid errors from imperfect washing of filters, the boiled product of digestion was filtered and 1000 cc. of the filtrate used in each determination. Where thymus nucleic acid and guanylic acid were employed the amount of the substance involved in any experiment was ascertained by an obvious calculation from the total amount employed. The hypoxanthine was weighed as nitrate and the free base calculated. Adenine was in no case obtained.

1. *Self-digestion of pig's pancreas 5 days at 40°. The numbers are milligrams.*

	GUANINE	XANTHINE	HYPOXANTHINE
Self-digestion	000	142	120
Self-digestion followed by acid hydrolysis	382	164	369
Hydrolysis of fresh gland	534		51 adenine 275

2. *The same.*

	GUANINE	XANTHINE	HYPOXANTHINE
Self-digestion	000	129	123
Self-digestion followed by acid hydrolysis	391	143	352

3. *The same.*

	GUANINE	XANTHINE	HYPOXANTHINE
Self-digestion	000	135	127
Self-digestion followed by acid hydrolysis	391	151	345

4. *The same.*

	GUANINE	XANTHINE	HYPOXANTHINE
Self-digestion	000	143	129
Self-digestion followed by acid hydrolysis	376	148	372

5. *The same with ox pancreas.*

	GUANINE	XANTHINE	HYPO-XANTHINE
Self-digestion	000	241	144
Self-digestion followed by acid hydrolysis	475	220	421

6. *Summary of the four experiments with pig's pancreas.*

	I	II	III	IV
Autolysis and hydrolysis guanine and xanthine	546	534	542	524
Hydrolysis of fresh gland. guanine	534			
Autolysis and hydrolysis hypoxanthine	369	352	345	372
Hydrolysis of fresh gland. adenine and hypoxanthine	326			

In the self-digestion of the pancreas, hydrolysis evidently occurs simultaneously in two ways. *In one way,* free purine bases are formed which are desamidized by the action of guanase and adenase long known to be present in this gland and the xanthine and hypoxanthine of self-digestion are thus produced. They should not necessarily be equal in amount nor bear any quantitative relation to one another; xanthine has always been found the greater of the two. *In the other way* phosphoric acid is split off giving rise to guanosine and adenosine. The former remains practically unaltered and by hydrolysis guanine is formed; while the latter is desamidized and by hydrolysis hypoxanthine is formed which appears as the increase of hypoxanthine when the self-digested product is hydrolyzed.

7. *Pig's pancreas and thymus nucleic acid. Each experiment involves 6 grams of this substance equivalent to 600 mg. of guanine and 540 mg. of adenine.*

	GUANINE	XANTHINE	HYPO-XANTHINE
Digestion at 40° 10 days	000	161	172
Digestion followed by acid hydrolysis	794	181	831
Digestion and hydrolysis control without nucleic acid	391	162	374
From the nucleic acid	403	19	457

8. *Ox pancreas and thymus nucleic acid. Each experiment involves 6 g. of the nucleic acid.*

	GUANINE	XANTHINE	HYPO-XANTHINE
Digestion at 40° for 10 days	000	276	189
Digestion followed by acid hydrolysis	869	278	860
Control. Digestion and hydrolysis without nucleic acid	469	214	402
From the nucleic acid	400	64	468

Thus from 6 grams of thymus nucleic acid were obtained 400 mg. of guanine and 468 mg. of hypoxanthine. Desamidization of the adenine group must have occurred in the digestion although phosphoric acid had been split off. *Surely, this is a demonstration of adenosine-desamidase in the practical absence of guanosine-desamidase.*

9. *Self-digestion of pig's liver 10 days at 40°.*

	GUANINE	XANTHINE	HYPO-XANTHINE
Self-digestion	210	210	000
Self-digestion followed by acid hydrolysis	192	445	000

PROCEEDINGS OF THE
AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

FIFTH ANNUAL MEETING.

New Haven, Conn., December 28–30, 1910.

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

ON THE PRESENCE OF A GLUCOSE-PROTEIN COMPOUND IN *ASCARIS LUMBRICOIDES.*

By FRANCIS H. McCRUDDEN.

(From the Rockefeller Institute for Medical Research, New York.)

Ascaris contains considerable quantities of a substance soluble in dilute alcohol which, on cleavage yields a sugar, a protein and a volatile substance. The sugar is probably glucose. The protein gives all the protein tests except the tests for tryptophan. It contains phosphorus and sulphur. The volatile substance is probably an aromatic oil.

THE ALBUMIN AND GLOBULIN IN THE OVARIES OF *BARBUS FLUVIATUS* AND THE PIKE.

By FRANCIS H. McCRUDDEN.

(From the Rockefeller Institute for Medical Research, New York.)

The albumin and globulin of *Barbus fluviatus* and the pike were isolated and examined chemically. All four gave positive biuret and xanthoproteic tests and contained phosphorus and sulphur. None of them contained iron. Both albumins gave a reducing substance on hydrolysis. The globulin did not contain any reducing substance. The albumin and the globulin of *Barbus fluviatus* gave a positive Millon's test, the albumin and globulin of the pike did not. Tests for tryptophan were negative in all four cases.

The absence of iron, reducing substance and bodies giving tryptophan tests show that the globulins of the eggs of these fish differ considerably from the globulins of the birds' eggs.

ON THE TOXIC ACTION OF CERTAIN FISH OVARIES.

By FRANCIS H. McCRUDDEN.

(From the Rockefeller Institute for Medical Research, New York.)

The ovaries of *Barbus fluviatus* and of the pike contain considerable amounts of a very toxic substance. Small quantities of the aqueous extract, injected subcutaneously into rabbits, cause severe convulsions. Death takes place in a few minutes, apparently from failure of the respiration. The toxic substance is not a simple chemical compound but is apparently of protein nature. The albumin and globulin were separated by dialysis and by dilution, and the toxic action appeared always in the albumin fraction.

SOME NEW TECHNIQUE FOR THE DETERMINATION OF TOTAL NITROGEN, AMMONIA AND UREA IN URINE.

By OTTO FOLIN, CHESTER FARMER, A. B. MACALLUM, and C. V. J. PETTIBONE.

(From the Laboratory of Biological Chemistry, Harvard Medical School.)

The new methods described for the determination of total nitrogen, ammonia and urea depend on the use of extremely small quantities (0.1—1.0 cc.) of urine. The ammonia formed is set free by an air current instead of by distillation and is subsequently estimated colorimetrically by means of Nessler's reagent and a colorimeter. By these methods the total nitrogen is determined in twenty minutes, the urea in about twenty-five minutes and the ammonia in about fifteen minutes.

THE SULPHUR BALANCE IN METABOLISM.

By ALONZO ENGLEBERT TAYLOR.

(From the Laboratory of Pathology of the University of California.)

The total sulphur in the diet, urine and feces of six normal men, covering periods of nearly three months, was determined in the attempt to fix the relations of the sulphur balance. The sulphur content of the foods and feces was determined by the peroxide method, as employed in the official method of the Department of Agriculture. The total sulphur in the urine was determined by a modification by Schmidt of the method of Benedict, checked with estimations by the peroxide method according to Folin. Applied to urines, the two methods give perfect agreement. An allowance of thirty milligrams of sulphur per day was made for the cutaneous elimination, it having been found in two tests that such an amount was daily eliminated through the skin.

When the figures are brought together, it is seen that no balance can be struck. The figures for the output are regularly and notably higher than those for the input. Since the subjects were known to be in normal nitrogen equilibrium, it is clear that the result is spurious. Since the total sulphur of the urine, when determined by two different methods, remains the same, the presumption follows that the figures for the input are wrong. It is easy to see wherein the method of combustion of the foods by peroxide might fail to give the total amounts of sulphur. It is difficult to see how the methods of determination of the sulphur in the urine and feces could give fictitiously high results. In short, we are driven to conclude that the figures for the input are too low, sulphur is lost in the processes of estimation. Apparently, the peroxide method, applied to foods, has not relieved us of our dependence upon the method of Carius. A few estimations with the bomb, using compressed oxygen, have given us higher figures than those obtained with the peroxide method. How these check up with those obtained by the method of Carius, we do not know. We shall report upon this next year.

THE OUTPUT OF AMMONIA IN NORMAL URINE.

By ALONZO ENGELBERT TAYLOR.

(From the Laboratory of Pathology of the University of California.)

The daily elimination of ammonia in six normal men, over periods of from one to three months each, was estimated by the method of Folin. When first undertaken, only 25 cc. of urine were used in the determinations. Since the amounts of ammonia recovered were very low, the amount of urine used was increased first to 50 and later to 100 cc. We employed a good stream of air, and the suction was continued for three hours. The removal of the ammonia, under such circumstances, follows the logarithmal curve. We used sodium carbonate for the alkalinisation, as advised by Folin. The apparatus was repeatedly checked up with the use of known amounts of $\frac{N}{10}$ ammonia, added under the precise conditions of dilution, time, etc. employed in the tests. The reliability of the apparatus, and the control of the conditions of estimation were in these check estimations shown to be absolute.

The results were very low. The usual figures for the ammonia nitrogen are given as from 0.3 to 0.5 gram per day. Our figures ran from 75 to 150 milligrams per day. Our urines were preserved on ice, without the use of any preservative except refrigeration. The lower results in our estimations, I am inclined to attribute to decomposition in the urine as commonly preserved with toluol, thymol or chloroform.

ADDENDUM IN PROOF. Two weeks' results in the estimations of ammonia, daily, in the refrigerated urines of eight normal men, in Philadelphia, has shown that the inference above stated was not correct. Using the method in the same way, except in one point, we are obtaining values running in the neighborhood of 300-400 milligrams per day. There is one difference in the method: we are using hydroxide, as recommended by Steel, instead of carbonate as advised by Folin. I doubt that this is the cause of the total difference. We shall make comparative tests to determine that. It is more likely that some factor in the diet or water of California, under the conditions of our experiment, was the cause of our abnormally low values. An excess of magnesium and preformed phosphoric acid might have resulted in the ammonia being eliminated as the triple phosphate in the feces.

ON CREATININE METABOLISM.

BY CARL VOEGTLIN AND C. TOWLES.

(From the Laboratory of Pharmacology of the Johns Hopkins University.)

In birds, creatine is an end product of metabolism. No creatinine is found in the urine by means of the picric acid reaction.

Creatine introduced is completely excreted. Creatinine, if fed, is excreted as such in the urine. There is no evidence of the presence of creatinase or of creatininase in the bird's organs.

In dogs, creatine fed or injected increases the output of creatinine in the urine. Dogs with Eck's fistulas show little if any difference from normal dogs as regards the creatinine metabolism. The liver can therefore hardly occupy an important place in creatinine metabolism as is claimed by some investigators.

MUCIC ACID AND CARBOHYDRATE METABOLISM.

BY LAFAYETTE B. MENDEL AND WILLIAM C. ROSE.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University.)

Feeding experiments on rabbits and dogs show that mucic acid in doses of 10 to 20 grams per day is not completely oxidized in the body, but appears unaltered in the urine in detectable amounts. Stoichiometrically equivalent amounts of lactose and galactose, introduced under exactly the same conditions, are completely oxidized without the appearance of a trace of mucic acid in the urine. These results indicate that mucic acid is presumably not an intermediary oxidative product in the metabolism of galactose or galactose-yielding carbohydrates. The urinary oxalic acid is only very slightly increased after the ingestion of large amounts of mucic acid. This increase is by no means as large as would be expected if mucic acid were a precursor of oxalic acid.

THE INFLUENCE OF ALCOHOL ON PROTEIN METABOLISM IN DOGS.[1]

BY WILLIAM SALANT AND J. B. RIEGER.

(From the Bureau of Chemistry, U. S. Department of Agriculture, Washington, D. C.)

This investigation was carried out on five dogs which received a mixed diet, containing protein equivalent to 0.7–0.8 gram of nitrogen per kilo. The caloric value of the food was 85.5 calories per kilo. Alcohol was given by mouth through a stomach tube or was fed with the food. The administration of 2–3.5 cc. of alcohol (diluted to 50 per cent) per kilo was followed by increased protein catabolism corresponding to a loss of 0.3–2.0 grams of nitrogen per day. Increased protein catabolism was also observed when such doses of alcohol were given with the food. The partition of nitrogen in the urine showed that total nitrogen and urea were increased in amount in three dogs, while in two they remained the same. Increased amounts of ammonia were also observed in some. Neither creatine or creatinine was appreciably effected. The output of purines precipitable by copper sulphate and sodium bisulphite showed a well marked rise. Smaller doses of alcohol (0.4–0.5 cc., diluted to 50 per cent) given with or without the food, exerted a sparing influence on protein. The nitrogen retained amounted to about 0.5 gram per day. The urinary nitrogen and urea were diminished. The other constituents, including purines were about the same as in normal conditions.

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[1] A preliminary communication, published by permission of the Secretary of Agriculture.

SYMPTOMS SHOWN BY PLANTS UNDER THE INFLUENCE OF DIFFERENT TOXIC COMPOUNDS.

By OSWALD SCHREINER.

(From the Bureau of Soils, U. S. Department of Agriculture, Washington, D. C.)

Observations made in connection with the effects of different organic substances in modifying and influencing growth of wheat seedlings has shown that toxic substances have a specific effect in modifying certain definite plant characteristics or functions. For instance, cumarin-affected plants have characteristic stunted tips and broad, distorted leaves. Vanillin strongly inhibits root growth. Quinone causes a tall and slender growth, with thin, narrow leaves, in strong contrast to the cumarin-affected plants. These various effects of toxic compounds are overcome by entirely different fertilizer combinations, the cumarin effect by phosphates, the vanillin effect by nitrates, the quinone effects by potassium salts. The absorption of salts while the plants are under the ininfluence of these toxic compounds is likewise different, thus showing that the effect of the poisons is deep-seated, influencing the entire metabolism of the plants. The changes brought about by etherization and the influence of certain gases in causing the so-called sleeping of carnations and other flowers belong to this class of phenomena.

Previous work with plants and toxic compounds has usually been made without the recognition that plants are affected in a markedly different manner by different poisons. The idea is developed that the plant, like the animal, shows characteristic symptoms with specific poisons, that there exists, as it were, a pharmacology of plants as well as of animals.

That organic substances can cause changes in certain definite plant characteristics, distortions, changed metabolism, etc., leads to the suggestion that harmful organic substances occurring in the soil or in the plant may be the direct or indirect cause of some now little understood plant physiological diseases.

A NEW PROCESS FOR THE PURIFICATION OF LIPINS, WITH DEMONSTRATIONS.[1]

By JACOB ROSENBLOOM and WILLIAM J. GIES.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

The authors have found that many ether-soluble substances, when dissolved in ether and placed in various permeable containers immersed in ether, readily pass through such membranes thus imposed. Similar results have been obtained with some of the same substances in other solvents. A demonstration was made of the ready diffusibility of the following substances in ether solution contained in *rubber* bags immersed in ether:

A. Lipochrome, fat, fatty acid, and cholesterol, from ether extract of egg yolk. (*There was no diffusion of lecithans.*).

B. Sudan III and brain cholesterol (artificial mixture).

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[1] This study is one of a projected series on *lipins*, which in turn constitutes a section of a comprehensive plan of research on the composition of protoplasm as well as the structural and dynamic relationships of cell constituents and products. These investigations are now in progress in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, and under the auspices of the George Crocker Special Research Fund.

C. Olive oil and lead oleate (artificial mixture).

The use of membranes of rubber, as was shown, offers numerous advantages in the separation and purification of various substances. The authors are now engaged in studies of the many possibilities which are suggested by the facts that were demonstrated.

AUTOLYSIS OF LIVER TISSUE AS AFFECTED BY THYROID ADMINISTRATION.

By ELIZABETHE COOKE and S. P. BEEBE.

(From the Laboratory of Experimental Therapeutics, Cornell University Medical College.)

The object of these experiments was to confirm the observations made previously by Schryver, namely, that thyroid administration would cause an increased rate of autolysis of the liver. For the purpose of these experiments the thyroid was administered in a variety of ways, the liver removed after a varying length of time and the autolysis measured in the usual manner and compared with controls. The results failed to confirm Schryver's conclusions. The autolytic rate in normal animals shows wide variations and no liver after a thyroid feeding showed a higher rate of autolysis than was attained from some normal livers without thyroid treatment.

In three cases a portion of the animal's liver was removed before thyroid feeding, the autolytic rate determined, the animal was fed on thyroid and the autolysis compared with the previous condition. In one case there was marked increase of the autolytic rate and in the two others, practically no change.

Administration of parathyroid, likewise, caused no demonstrable change in the rate of autolysis.

THE NATURE OF THE CHEMICAL COMBINATIONS OF POTASSIUM IN THE TISSUES.

By W. KOCH and C. C. TODD.

(From the Laboratory of Biochemistry and Pharmacology, University of Chicago.)

Some investigations with F. H. Pike on the proportion of anions and kations in the nervous system indicated a greater amount of potassium and sodium than could be accounted for by the chloride, sulphates and phosphates present. The discovery of potassium and sodium phosphatid combinations, already predicted by the work of Thudichum, has served in a large measure to account for this discrepancy.

The present communication is in the nature of a preliminary statement regarding the extension of this work to other tissues of the body, which has already been begun but will not be published in full until a greater amount of analytical results have been obtained. The results so far indicate that such sodium and potassium phosphatid compounds exist in all the tissues of the body and are probably of much more importance than the hitherto assumed ion protein combination.

THE ALLANTOIN-PURINE EXCRETION OF THE MONKEY.[1]

By ANDREW HUNTER and MAURICE H. GIVENS.

(From the Department of Physiology and Biochemistry, Cornell University, Ithaca, N. Y.)

From 75 cc. of the urine of a monkey upon a meat-free diet were isolated 8.5 mgm. of allantoin in typical crystals melting at 231° C. From 500 cc. of the mixed urine of two monkeys we obtained 172 mgm. of crystalline, though not entirely pure allantoin. In other 500 cc. of urine from the same two animals we could detect no uric acid, and only 4.5 mgm. of nitrogen in the form purine bases. Of a third mixed sample two portions of 200 cc. each were taken. From the one we isolated 66.1 mgm. of pure allantoin (m. p., 230° C.); the other contained 3.2 mgm. of purine nitrogen. Of the total allantoin-purine nitrogen in this sample 88.1 per cent therefore was in the form of allantoin. It follows that the purine metabolism of our animals is the of same type as that of the lower mammals, and does not in the least resemble that of man.

These findings confirm the single observation of Wiechowski,[1] and support the general conclusion to which Wells,[2] in spite of failure to isolate allantoin from the urine, was led by a study of the enzyme equipment of monkey tissues.

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[1] Wiechowski: Hofmeister's *Beitrage*, xi, p. 101, 1908.

[2] Wells: *Journ. of Biol. Chem.*, vii, p. 171, 1910.

In future work we propose to determine the extent of the monkey's daily allantoin excretion, and the effect upon it of purine feeding and other experimental conditions.

BIOLOGICAL ANALOGIES IN SOIL OXIDATION.

By OSWALD SCHREINER and M. X. SULLIVAN.

(From the Bureau of Soils, U. S. Department of Agriculture, Washington, D. C.)

The soil is the seat of many biochemical activities which directly or indirectly affect soil fertility. Many of the processes in the soil are analogous to those occurring in plants and animals. Soils may show fatigue under a one crop system and likewise under unsanitary conditions contain material which is retardative of plant growth. Many other compounds, some of which are known to be products of proteolytic digestion, occur in soils. The soil *per se* has oxidizing and catalyzing powers which in cropped soils are due partly to activities of plant roots, but in air-dried soils are due mainly to non-enzymotic soil constituents, inorganic and organic, working separately, conjointly or in reinforcing and activating combinations. The recently discovered activating action of salts of organic hydroxy-acids and the discovery that alfalfa laccase is a mixture of salts of organic hydroxy-acids have a close counterpart in soil oxidation studies.

ADDITIONAL EXPERIMENTS WITH THE BIURET REAGENT.[1]

By J. L. KANTOR and WILLIAM J. GIES.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

Further study of the biuret reagent has emphasized many advantages that result from its employment.[1] Its general utility was demonstrated.

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[1] This study is one of a projected series on *proteins and their combining qualities*, which in turn constitutes a section of a comprehensive plan of research on the composition of protoplasm as well as the structural and dynamic relationships of cell constituents and products. These investigations are now in progress in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, and under the auspices of the George Crocker Special Research Fund.

The authors have found that when the reagent is filtered through paper or cotton wool, the copper may be wholly removed from the solution. Filter paper, parchment paper, cotton wool and other forms of cellulose, when immersed in the reagent, absorb copper and, if present in sufficient quantity, completely decolorize the liquid. A blue copper-cellulose product, apparently a compound, results. This product does not form in the presence of a chemical excess of glycerol or protein.

Filter paper which, by immersion in the reagent, has been converted into the copper-cellulose product, may be used, either wet or dry, for the detection of protein. Cut into strips, the prepared material resembles blue litmus paper strips, and when dipped into a neutral or alkaline protein-containing solution or, if wet, when brought into contact with a neutral or alkaline protein-containing powder, the blue color is promptly turned to red.

The reagent is useful for the detection of reducing substances. Parchment paper, if kept in the reagent for some time, absorbs the copper and yields the blue cellulose product, but this compound(?) is gradually decomposed by reduction, a loose red precipitate, apparently of cuprous oxide, is spontaneously produced, and the parchment, if present in large proportion, returns, in part at least, to the original colorless condition and the fluid loses all its blue color. Parchment paper exerts similar effects on the Fehling and Fehling-Benedict reagents.

The colors obtained with given volumes of the biuret reagent and a protein solution gradually fade. In a few months the difference in any instance (compared with freshly prepared controls) is quite marked. Such color standards are reliable during shorter periods, however. The reagent may therefore be used satisfactorily in a number of types of colorimetric quantitative determinations of protein. The study is in progress.

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[1] Gies: *Journ. of Biol. Chem.*, 1910, vii, Proceedings of the American Society of Biological Chemists, p. lx. The alkali in the reagent should amount to about 10 per cent. A typographical error in the original published reference to the reagent indicated 1 per cent as the quantity of alkali to be employed. This error was corrected in the official proceedings of the Society, where NaOH was also substituted for KOH, for economic reasons. (*Proceedings of the American Society of Biological Chemists*, 1910, i, p. 273.)

CHEMICAL STUDIES OF HUMAN SWEAT.

By LOUIS W. RIGGS, (by invitation).

(From the Laboratory of Experimental Therapeutics, Cornell University Medical College.)

Samples of sweat were collected by placing the patient without clothing in a rubber bag which inclosed the entire body except the head, and sweating induced by covering with hot blankets.

Forty-five samples were examined; the total nitrogen, nitrogen as urea plus ammonia, inorganic solids, potassium and chlorine were determined in a majority of the samples. Nitrogen as ammonia was determined in six samples from normal persons, and calcium in seven samples from nephritics.

The sweat from certain nephritics did not differ greatly from that of normal persons. Other nephritics eliminated twice or three times as much nitrogen and the same cases twice as much much potassium and less than one-half as much inorganic solids and chlorine per 100 cc. of sweat as normal individuals.

ON THE RECOVERY OF ADENINE.

By G. DeF. BARNETT and WALTER JONES.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

To free adenine from hypoxanthine the base must be converted into the picrate, a compound which as such is useless and can be changed into a non-toxic salt only by the use of an excessive amount of ether. However, when the picrate is dissolved in ammonia and the solution treated with ammoniacal silver nitrate the adenine is quantitatively precipitated while, contrary to what might be expected, more than 90 per cent of the picric acid remains dissolved. This forms the basis of a method of recovering adenine and of dealing with filtrates from adenine picrate which contain other purine bases with picric acid.

THE UTILIZATION OF INGESTED FAT UNDER THE INFLUENCE OF COPIOUS AND MODERATE WATER DRINKING WITH MEALS.

By H. A. MATILL and P. B. HAWK.

(From the Laboratory of Physiological Chemistry of the University of Illinois.)

Experiments were performed upon men living on a uniform diet; a preliminary period of small water ingestion was followed by a period of large water ingestion, and this, in turn, by a final period with the original conditions. The determination of fat in feces was made by the Kumagawa-Suto method and was entirely satisfactory. When one liter of water was taken with each meal, the average daily excretion of fat was much reduced and a similar but less marked reduction was observed when 500 cc. of water were taken.

The better digestion and absorption of fat was probably due to any or all of the following factors: 1, increased secretion of gastric juice and, independently, of pancreatic juice; 2, increased acidity of the chyme bringing about more active secretion of pancreatic juice and bile; 3, increased peristalsis due to larger volume of material in the intestine; 4, increased blood pressure due to rapidly absorbed water; 5, more complete hydrolysis by lipase because of increased dilution.

A METHOD FOR THE QUANTITATIVE DETERMINATION OF FECAL BACTERIA.

By H. A. MATTILL and P. B. HAWK.

(From the Laboratory of Physiological Chemistry of the University of Illinois.)

By a method of fractional sedimentation, an adaptation of MacNeal's modified Strasburger method, the bacterial portion of the feces is separated from the other solid material. By the Kjeldahl method the nitrogen of this bacterial substance is determined directly instead of making this determination upon the dried bacteria after absolute alcohol and ether extraction. In men on a uniform diet containing negligible quantities of cellulose bacterial nitrogen comprised 54 per cent of the total fecal nitrogen, a value 7 per cent higher than heretofore found. This higher and probably more accurate value may be a result of the omission of the absolute alcohol and ether extractions. In addition, a saving of about five days' time and of considerable labor, is accomplished by that omission.

ON THE DIFFERENTIAL LEUCOCYTE COUNT DURING PROLONGED FASTING.

By PAUL E. HOWE and P. B. HAWK.

(From the Laboratory of Physiological Chemistry of the University of Illinois.)

Blood smears were examined from a dog fasting 117 days; from a dog during two fasts of 15 and 30 days respectively, each of which continued to the inception of the premortal rise; from an anæmic dog, fasting 48 days; and from a puppy, one month old fasting seven days. Smears were also taken from each of two men, during a fasting period of seven days and a subsequent regeneration period of eight days. The smears were stained by a method devised by MacNeal.

From the data we conclude that as a result of fasting in the normal dogs there was a decrease in the number of polynuclear leucocytes with an increase in the number of small mononuclear lymphocytes. There were no marked changes in the other forms of cells. In the anæmic dog, the number of polynuclear leucocytes increased while the number of small lymphocytes decreased. A condition of eosinophilia existed at the beginning of the fast which disappeared as the fast progressed. In men there was an increase in the number of polynuclear leucocytes at the beginning of the fast followed by a decrease below normal at the end of seven days. During a subsequent feeding period the cells tended to assume their normal relations.

ON THE CATALASE CONTENT OF TISSUES AND ORGANS AFTER PROLONGED FASTING.

By P. B. HAWK.

(From the Laboratory of Physiological Chemistry of the University of Illinois.)

The study embraced the examination of the tissues and organs of four dogs which were subjected to periods of fasting ranging from 7 to 104 days. A pup one month old was subjected to a 7 day fast, a dog from 1 to 2 years old served as the subject of the 30 day fast, whereas the longer fasts were carried out upon mature animals. The dogs were fed a constant water ration, water being introduced by means of a stomach tube.

At the termination of the fasting periods chloroform-water extracts of the tissues and organs were prepared and their catalase values determined. The tissues and organs of normally nourished dogs were subjected to a similar examination in order to secure data for comparative purposes. The method employed was the measurement of the volume of oxygen liberated from 5 cc. of neutral hydrogen peroxide (Dioxygen) by 0.25 cc. of the extract acting through an interval of two minutes. An apparatus, the principle of which has been described by Bryan[1] was utilized in the measurement of the liberated oxygen.

An examination of the data indicates the catalase values of the tissues and organs are much lower, in every instance. than those of the normal tissues and organs. It was also observed that the order of the tissues when arranged according to their catalase content was distinctly altered in the fasting animals from the order in force under normal conditions. There was apparently no uniformity as to the specific alterations which took place in the catalase content of animal tissues and organs under the influence of fasting. The data obtained from the four fasting animals under consideration were in every case different from normal catalase values, but at the same time these catalase values obtained from fasting animals exhibit marked variations when we make a comparison of the data from the four animals under investigation. It is of particular interest that the tissues and organs of the dog which was subjected to the most prolonged period of fasting exhibited less alteration from the normal than did the tissues and organs of those animals which were subjected to much shorter fasts.

THE RELATION OF THE ADRENALS TO TUBERCULIN POISONING.

By JAMES P. ATKINSON AND CHAS. B. FITZPATRICK.

(From the Chemical and Research Laboratories, Department of Health, City of New York.)

We have recently found that commercial adrenalin preparations and saline extracts of the freshly removed dog's adrenal gland prevented a depression when mixed with tuberculin and the mixture injected intravenously, thus showing an antagonistic action between tuberculin and the adrenals. The intravenous injection of samples of tuberculin containing the active depressor substance, after the adrenals had been removed, caused a drop with a more gradual recovery of the previous pressure level, than when the adrenals had been left intact.

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[1] Bryan: *Journ. of the Amer. Chem. Soc.*, xxviii, p. 28, 1906.

These and our other published results indicate:

A. That tuberculin is a complex substance consisting of at least two poisons, one a blood depressor destroyed by heat and antagonized by adrenalin, and the other a substance characterized by its fatal effects on tuberculous guinea pigs when injected subcutaneously. This toxicity resists heating to 110° C. and is not antagonized by adrenalin.

B. It is possible that we have evidence here that in the absence or the abnormal diminution of the adrenal secretion, extracts of the tubercle bacillus are more toxic than otherwise and that their administration mixed with the whole adrenal or some part of it, in a measure overcomes their deleterious action.

C. It is possible that the neutralizing value of blood mixed with tuberculin or some other appropriate adrenal antagonist might be a valuable index of the functional condition of the adrenal glands.

PROTEIN AS A FACTOR IN THE NUTRITION OF ANIMALS: I. A STUDY OF THE PHYSICAL CONSTANTS OF FATS FROM SWINE.

BY A. D. EMMETT AND E. C. CARROLL.

(From the Laboratory of Physiological Chemistry, Department of Animal Husbandry, University of Illinois.)

In this experiment, Berkshire pigs of known ancestry and age were fed different amounts of a highly nitrogenous feed—bloodmeal. In conjunction with this a basal feed of ground corn was used. Further, crude calcium phosphate was so fed that all the animals got approximately the same amount of phosphorus in the ration. Nine pigs were used, these being divided into three lots of three pigs each. Lot I was fed on the low protein plane; Lot II, on the medium or balanced plane, and Lot III on the high plane. It was the plan to kill the two most representative pigs out of each lot. However, in the case of Lot I, only one pig lived to the end of the experiment. The five animals were subjected to a most thorough slaughter test. Of the various parts of the entire carcass that were studied chemically, this paper has to deal with leaf fat, the back fat, and a composite sample of the leaf, back, intestinal, and jowl fats. The following determinations were made: specific gravity, saponification number, soluble acids, insoluble acids, volatile acids, free fatty acids, iodine number, melting point and index of refraction. The technic of the methods was carefully worked out in advance and the limits of error ascertained for each determination.

It was found from this preliminary study of the influence of protein feed:

1. That if the ancestry, age, and type of the animals are not considered in comparing the data, the different amounts of protein feed have no apparent influence on the physical constants of the fats. The individual idiosyncrasies of the animals may be as great a factor or greater than that of feed.

2. That, if litter mates be compared, of which there was one in Lot I comparable with one in Lot II, and one in Lot II comparable with one in Lot III, these data show that the differences in the physical constants due to feed were very slight. Here, however, the matter of individuality again may be the controlling factor.

3. That, if the data from the various samples be compared with respect to the *kind* of fats, they show that the values for the iodine number and melting point are quite different in the case of the back fat when compared with the leaf and composite samples of fat. The averages for the iodine number are 51.23 for the back fat, 45.60 for the leaf fat, and 45.91 for the composite fat. The averages of the melting point determinations for the back, leaf, and composite samples of fat are respectively: 34.1, 42.8, and 45.7 C.

4. That, comparing all samples of fat in respect to both the protein-feed and the kind of fat and without regard to ancestry, age, and type of the animal, or to individuality, the specific gravity, saponification number, the insoluble acids and the index of re-

fraction appear to be practically constant in each case, averaging for all the samples, 0.8934, 196.94, 95.58, and 1.4595 respectively.

ON INOSINIC ACID.

By W. A. JACOBS and P. A. LEVENE.

(From the Rockefeller Institute for Medical Research, New York.

The results of our previous work upon the constitution of inosinic acid have demonstrated it to be a hypoxanthin phosphoribosid. There still remained the question of the position of the phospho-group in the ribose molecule and also that in the hypoxanthin molecule which functions in the union with the sugar. In the present work we have been able to prove that the phosphoric acid sits on the ω-C atom of the ribose. After oxidation with nitric acid of ribose phosphoric acid obtained from inosinic acid according to the usual procedure employed for obtaining dicarboxylic acids, we succeeded in obtaining as the pure Ca salt an acid which analysis and chemical properties demonstrated to be a phospho-ribonic acid. After cleavage of the phosphoric acid by neutral hydrolysis, ribonic acid was obtained as the pure crystalline Cd salt. The yield of phospho-ribonic acid was approximately 75 per cent of the theory reckoned from inosinic acid. The remaining 25 per cent was accounted for by the free H_3PO_4 formed by cleavage during oxidation. Attempts to isolate any phosphotrioxyglutaric acid, which would be formed if the phosphoric acid were on any other than the end C-atom proved fruitless. These facts demonstrate that the phospho group is bound on the ω-C atom and prevents further oxidation of the phospho-ribonic acid to a trioxyglutaric acid derivative.

ON THE YEAST NUCLEIC ACID.

By P. A. LEVENE and W. A. JACOBS.

(From the Rockefeller Institute for Medical Research.)

From previous work the present authors were led to the conclusion that the molecule of the yeast nucleic acid was composed of simple neucleotides, which consisted of phosphoric acid conjugated either with a purin-ribosid or with a pyrimidin complex. This assumption was supported by the discovery among the products of partial hydrolysis of two ribosids—guanosin, adenosin and of the cytidin complex—cytosin. The authors succeeded in isolating a substance which possessed the properties and the composition of an equimolecular mixture of cytidin and uridin nucleotides. The substance did not contain purin bases, on further hydrolysis failed to reduce Fehling's solution, formed furfurol on distillation with hydrochloric acid, contained amid nitrogen and total nitrogen in proportion 1:5, on hydrolysis gave rise to uracil and cytosin.

A NEW MICROSCOPIC TEST FOR FREE ACID.[1]

By J. L. KANTOR AND WILLIAM J. GIES.

(*From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.*)

When collagen fibers are immersed in dilute solutions of various mineral or organic acid solutions the fibers quickly swell and become transparent. We hope to learn later the true *chemical* nature of this well known process. *When, however, the acid in a solution possessing this power is combined with protein such swelling does not take place.* Either of these observations can readily be made through a microscope with a single tendon fibril and a drop of acid solution. Proportions of *mineral* acids which do not respond to the Töpfer or Günzberg test appear to be devoid of prompt swelling effect on individual collagen fibers. *Various proportions of organic acids (e.g., acetic and lactic) which cannot be detected with the above named reagents, immediately effect a swelling of collagen fibers,* the change being strikingly obvious when the test is made microscopically. Similar results have been obtained with fibrin. A detailed study of the test is in progress.

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[1] This study is one of a projected series on *proteins and their combining qualities*, which in turn constitutes a section of a comprehensive plan of of research on the composition of protoplasm as well as the structural and dynamic relationships of cell constituents and products. These investigations are now in progress in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, and under the auspices of the George Crocker Special Research Fund.

CHANGES IN THE BLOOD AND MUSCLE FOLLOWING BILATERAL NEPHRECTOMY AND DOUBLE URETERAL LIGATION.

By HOLMES C. JACKSON.

(From the Laboratory of Physiology of the New York University and Bellevue Hospital Medical College.)

1. When all the various conditions of the experiment (diet, lack of infection, etc.) remained constant there appeared to be a slight tendency on the part of the animals with both kidneys removed to survive the operation for a longer period than was the case in double ureteral ligation. The analyses of blood following both operative procedures showed no differences which could account for this.

2. The molecular concentration (Δ) of the blood increased after both operations until death. The electrical conductivity (K) and chloride content of the blood after a temporary rise on the day following the operation progressively decreased. Hence the rise in concentration was brought about by an increase in organic substances mainly nitrogenous and non-coagulable in character as was shown by the blood analysis.

3. Following both operations the blood becomes more venous with a resulting increased viscosity due to CO_2 and the time of coagulation is increased.

4. No vicarious elimination of uric acid or purine bases occurs into the intestine.

5. Hydremia apparently does not take place as the result of either operation; and in only two cases with double ureteral ligation was subcutaneous edema present. In four instances where the ureters were tied the animals developed true uremic convulsions.

6. Analysis of the muscle indicated that following double nephrectomy there existed a tendency for the tissue to lose water.

THE INFLUENCE ON METABOLISM OF OILS INJECTED SUBCUTANEOUSLY AND INTRAVENOUSLY.

By JOHN R. MURLIN and LLOYD H. MILLS.

(From the Physiological Laboratory of the Cornell University Medical School, New York City.)

A preliminary report on this work has already been made.[1]

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[1] *Proc. Soc. for Exp. Biol. and Med.*, vii, p. 166, 1910.

Additional experiments on dogs and on a tuberculous man were presented at this meeting. One dog was given a basal diet containing 0.4 gram of nitrogen and 52 calories of energy per kilogram per day. Twenty grams of peanut oil superimposed on this by mouth for three days had exactly the same effect on the amount of nitrogen excreted and on the amount of fat in the feces as 20 grams of lard. Injected subcutaneously, the oil was a little less favorable. The same experiment was repeated with the same result by superimposition on a basal diet containing the same quantity of nitrogen and 60 calories per kilogram.

The man was a typical case of grave pulmonary tuberculosis on the right side. He was placed for ten days on a basal diet of milk, cream and eggs, containing 17 grams of nitrogen and 44 calories of energy per kilogram. When 21 grams of peanut oil were injected subcutaneously for three days it produced a slightly less favorable effect on the nitrogen balance than when the same amount of oil in the form of Russell's Emulsion was given by mouth, but more favorable than when no oil was given. When 95 grams of peanut oil were injected daily for five days the output of nitrogen was greater than when an isodynamic quantity of lactose was added to the diet.

Preliminary experiments only on the use of emulsions intravenously were reported. As much as 40 cc. of a fine emulsion of peanut oil using 5 per cent lecithin as emulsifier, can be given to a dog of 10 kilos in this way without any respiratory distress. This emulsion also offsets the influence of various narcotics, *e.g.*, ether, morphine. Its action may, however, be due entirely to the lecithin as already observed by Nerking.[2]

ON A SPECIFIC NUCLEASE FOR GUANYLIC ACID.

By WALTER JONES.

(From the Laboratory of Physiological Chemistry of the Johns Hopkins University.)

Pigs' pancreas will not split off guanine from added guanylic acid nor even decompose the guanylic acid of the tissue itself in autodigestion. But beef's spleen decomposes added guanylic acid giving rise to a mixture of xanthine and uric acid. This action of the spleen cannot be due to the presence of nuclease, for nuclease is present in the pancreas.

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[2] *Münch. Med. Woch.*, 1909, No. 29, p. 1475.

THE STIMULATION OF GASTRIC SECRETION UNDER THE INFLUENCE OF WATER DRINKING WITH MEALS.

BY F. WILLS AND P. B. HAWK.

(From the Laboratory of Physiological Chemistry of the University of Illinois.)

Two men brought to nitrogen equilibrium through the ingestion of a uniform diet of low water content were caused to drink, *at meal time,* extra volumes of water ranging from 1500 cc. to 4000 cc. per day. During the periods of increased water ingestion there was in each instance an increase in the ammonia excretion which was *directly proportional to the extra volume of water ingested.* This finding was interpreted as indicating that the water ingestion had stimulated the flow of gastric juice, thus causing the production of a greater quantity of hydrochloric acid than could be neutralized by the customary means. A certain part, at least, of the excess acid was therefore, subsequently neutralized by ammonia which had been produced from the deamidation of protein material and which under ordinary conditions would have been utilized in the formation of urea. This excess acid would consequently probably appear, for the most part, in the urine as ammonium chloride. The uniform relationship between the water ingestion and the ammonia output might perhaps be considered as indicating that there was an attempt on the part of the gastric cells to maintain a *uniform acid concentration.* If we calculate the increased ammonia excretion, on the basis of a 100 cc. increase in the water ingestion we find that the excretion was a trifle higher during moderate water drinking than during copious water drinking. This would indicate that after a certain limit had been reached in water ingestion each succeeding 100 cc. of water was less efficient as a stimulating factor than were the 100 cc. portions ingested before the limit above mentioned had been reached. The ratios for the two subjects are appended:

SUBJECT W.		SUBJECT E.	
EXPERIMENT I.	EXPERIMENT II.	EXPERIMENT III.	EXPERIMENT IV.
$\frac{\text{Copious } H_2O}{\text{Moderate } H_2O}$	$\frac{NH_3 \text{ Inc. (Cop.)}}{NH_3 \text{ Inc. (Mod.)}}$	$\frac{\text{Copious } H_2O}{\text{Moderate } H_2O}$	$\frac{NH_3 \text{ Inc. (Cop.)}}{NH_3 \text{ Inc. (Mod.)}}$
1:1.81	1:1.75	1:2.66	1:2.54

Increased NH_3 per 100 cc. H_2O (grams)

3000 cc. H_2O	1500 cc. H_2O	4000 cc. H_2O	1500 cc. H_2O
0.0071	0.0081	0.0039	0.0040

The Utilization of the Mono-α-amino-acids and the Polypeptides by the Tubercle Bacillus. By Wm. F. Koelker and Ben W. Hammer.

A New Synthetic Tuberculin. By Wm. F. Koelker and Ben W. Hammer.

The Sources of Error in the Folin Method for the Estimation of Creatinine. By A. E. Taylor, (*Journal of Biological Chemistry*, ix, p. 19, 1911.).

The Gaseous Metabolism of the Heart. By T. Gregor Brodie.

Hydrolysis of Casein. By Thomas B. Osborne and H. H. Guest.

A Contribution to the Chemistry of Invertase. By A. P. Mathews (*Journal of Biological Chemistry*, ix, p. 29, 1911.).

The Relation of the Adrenals to the Tolerance of Air in the Circulatory Apparatus. By J. P. Atkinson and C. B. Fitzpatrick.

The Action of Plasma of Animal Organs and of Blood Serum on Inosin, Cytidin, Inosinic Acid and Guanylic Acid. By P. A. Levene and F. Medigreceanu (*Journal of Biological Chemistry*, ix, p. 65, 1911.).

On the Combined Action of Muscle Plasma and Pancreatic Extract on Glucose and Maltose. By P. A. Levene and G. M. Meyer (*Journal of Biological Chemistry*, ix, p. 97, 1911.).

Some illustrations in Animal and Vegetable Cells of the Gibbs-Thomson Law of the Surface Condensation of Solutes. By A. B. Macallum.

ON THE REFRACTIVE INDICES OF SOLUTIONS OF CERTAIN PROTEINS.

V. GLIADIN.

By T. BRAILSFORD ROBERTSON and J. E. GREAVES.

(From the Rudolf Spreckels Physiological Laboratory of the University of California.)

(Received for publication, February 28, 1911.)

In previous communications it has been shown by one of us[1] that if proteins (casein, ovomucoid, ovovitellin, paranuclein, serum globulin) be dissolved in alkaline or acid aqueous solutions the change in the refractive index of the solvent is directly proportional to the concentration of the protein which is dissolved therein, and that, when allowance is made for the alteration in the refractive index of the solvent with temperature, this change is independent of the temperature between 20° and 40° C. It has also been shown that solutions of serum globulin can be obtained in alkaline alcohol or acetone-water mixtures and of casein in alkaline alcohol-water mixtures and that the influence which these proteins exert upon the refractive indices of these solvents differs with the nature and composition of the solvent. It appeared of interest to further investigate the influence of the nature of the solvent upon the power of dissolved protein to change its refractive index. For this purpose the alcohol-soluble protein of wheat, gliadin, offers exceptional advantages since it is known to be soluble in a large variety of solvents.[2]

The gliadin was prepared by the following method, based upon that employed by Osborne and Harris.[3]

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[1] T. Brailsford Robertson: *Journ. of Physical Chem.*, xiii, p. 469, 1910. This *Journal*, vii, p. 359,1910; viii, pp. 287, 441, 507, 1910.

[2] G. W. E. Mathewson: *Journ. Amer. Chem. Soc.*, xxviii, p. 1482, 1906.

[3] T. B. Osborne and I. F. Harris: *Amer. Journ. of Physiol.*, xvii, p. 223, 1906.

> Gluten was prepared by kneading dough, made from wheat flour, in a stream of cold water until all the starch had been washed out; it was then partially dried and a moisture determination was made. The moist gluten thus obtained was finely chopped and mixed with twenty times its weight of alcohol of such a strength that with the water in the gluten it formed an alcoholic solution containing 70 per cent of alcohol by volume. The mixture of gluten and 70 per cent alcohol was then allowed to stand, with frequent shaking, for 48 hours. After allowing the mixture to settle for 10 hours the supernatant alcoholic solution of gliadin was syphoned off and filtered through very finely shredded and well-washed and dried asbestos until a perfectly clear filtrate was obtained. The filtrate was then evaporated, under a pressure of about one-half an atmosphere, until frothing prevented further concentration. It was then cooled and very slowly poured, with constant stirring, into about one hundred times its volume of ice-cold distilled water, containing 10 grams of sodium chloride per liter. The gummy mass, which usually collects on the stirring rod, was dissolved by the addition of the smallest possible amount of absolute alcohol, and this solution was evaporated, under reduced pressure, to a thick syrup; the syrup was then cooled and poured, in a very fine stream, with constant stirring, into hot absolute alcohol. The precipitate was dissolved in 70 per cent alcohol and this solution was evaporated under reduced pressure, with the occasional addition of absolute alcohol, until a thick syrup was again obtained. The gliadin was precipitated from this syrup by the method just described washed three times with ether (*über Natrium destilliert*), partially dried over sulphuric acid, ground up as finely as possible, and then completely dried over sulphuric acid, in partial vacuum, at room temperature.

The gliadin thus prepared, after passing through a fine sieve, is obtained as a loose white powder.

Two series of solutions were prepared, the one containing 2 grams and the other 1 gram of gliadin per 100 cc. of solvent. The solvents employed were 60 per cent and 70 per cent ethyl alcohol (Kahlbaum, C. P.), 25 per cent and 50 per cent acetone (highest purity, Merck), 60 per cent propyl alcohol (highest purity, Merck), 75 per cent phenol (Kahlbaum's synthetisch, C. P.), $\frac{N}{10}$ acetic acid and $\frac{N}{10}$ potassium hydroxide. The percentages are percentages by volume and the alcoholic, phenol and acetone solutions were standardised by specific gravity determinations. In 60 per cent and 70 per cent ethyl alcohol a very small proportion (less than 0.2 per cent of the dry substance) of the gliadin was insoluble. In 25 per cent acetone the solution containing 2 per cent of gliadin contained also a slight insoluble residue; in the remainder of the solvents the gliadin dissolved completely, forming clear solutions.

The refractive-index determinations were made in a Pulfrich refractometer reading correctly to within 1′ of the angle of total reflection; a sodium flame was employed as the source of light.

The following were the results obtained. The values headed "*a*" are calculated from the formula, $n - n_1 = a \times c$ where n is the refractive index of the solution, n_1 that of the solvent and c is the percentage of gliadin in the solution.

SOLVENT	TEMPERATURE	GRAMS OF GLIADIN IN 100 CC. OF SOLVENT	REFRACTIVE INDEX OF SOLUTION	$a = \frac{n - n_1}{c}$
$\frac{N}{10}$ Acetic acid	25°	0	1.33426	——
$\frac{N}{10}$ Acetic acid	25°	1	1.33592	0.00166
$\frac{N}{10}$ Acetic acid	25°	2	1.33743	0.00159
$\frac{N}{10}$ KOH	20°	0	1.33537	——
$\frac{N}{10}$ KOH	20°	1	1.33703	0.00166
$\frac{N}{10}$ KOH	20°	2	1.33871	0.00162
60 per cent Ethyl Alcohol	25°	0	1.36190	——
60 per cent Ethyl Alcohol	25°	1	1.36333	0.00143
60 per cent Ethyl Alcohol	25°	2	1.36477	0.00144
70 per cent Ethyl Alcohol	25°	0	1.36450	——
70 per cent Ethyl Alcohol	25°	1	1.36605	0.00155
70 per cent Ethyl Alcohol	25°	2	1.36750	0.00150
60 per cent Propyl Alcohol	25°	0	1.37171	——
60 per cent Propyl Alcohol	25°	1	1.37329	0.00158
60 per cent Propyl Alcohol	25°	2	1.37468	0.00149
25 per cent Acetone	25°	0	1.34821	——
25 per cent Acetone	25°	1	1.34983	0.00162
25 per cent Acetone	25°	2	1.35164	0.00172
50 per cent Acetone	25°	0	1.36082	——
50 per cent Acetone	25°	1	1.36235	0.00153
50 per cent Acetone	25°	2	1.36369	0.00144
75 per cent Phenol	22°	0	1.49649	——
75 per cent Phenol	22°	1	1.49611	−0.00038
75 per cent Phenol	22°	2	1.49573	−0.00038

It will be observed that in the less refractive solvents the introduction of gliadin increases the refractive index, but that in the most highly refractive solvent, namely 75 per cent phenol, the addition of gliadin *decreases* the refractive index.

In order to obtain a satisfactory estimate of the probable degree of accuracy of these determinations it is necessary to recollect that each determination of the angle of total reflection is liable to an error of 1′. An error of 1′ in the determination of the angle of total reflection corresponds to an error of about 0.00009 in the determination of the refractive index. Since the absolute error in each determination of $n-n_1$ is the same, the error in the determination of a must be less in proportion to the magnitude of c. In order to assign to each determination its due weight in the estimation of the mean value of a for any solvent we must therefore add together all of the observed values of $n-n_1$ and divide this sum by the sum of the concentrations employed.[1]

Proceeding in this way we obtain the following values of a.

Solvent	$a=$
$\frac{N}{10}$ Acetic acid	$-\ 0.00161 \pm 0.00006$
$\frac{N}{10}$ KOH	$-\ 0.00167 \pm 0.00006$
60 per cent Ethyl alcohol	$-\ 0.00143 \pm 0.00006$
70 per cent Ethyl alcohol	$-\ 0.00152 \pm 0.00006$
60 per cent Propyl alcohol	$-\ 0.00152 \pm 0.00006$
25 per cent Acetone	$-\ 0.00168 \pm 0.00006$
50 per cent Acetone	$-\ 0.00147 \pm 0.00006$
75 per cent Phenol	$-\ 0.00038 \pm 0.00006$

CONCLUSIONS.

1. The value of a, in the equation $n-n_1 = a \times c$ where n is the refractive index of the solution of the protein, n_1 that of the solvent and c is the percentage concentration of the protein, has been determined for gliadin in various solvents.
2. In the less highly refractive solvents the introduction of gliadin increases the refractive index; but in the most highly refractive solvent employed, namely 75 per cent phenol, the addition of gliadin *reduces* the refractive index.

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[1] Cf. T. Brailsford Robertson: This *Journal*, viii, p. 507, 1910.

A METHOD FOR QUANTITATIVE DETERMINATION OF ALIPHATIC AMINO GROUPS.

APPLICATIONS TO THE STUDY OF PROTEOLYSIS AND PROTEOLYTIC PRODUCTS.

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(Received for publication, March 6, 1911.)

It has long been known that aliphatic amino groups react with nitrous acid according to the equation

$$R.NH_2+HNO_2=ROH+H_2O+N_2$$

Since the nitrogen in gaseous form leaves the system, the reaction should theoretically proceed quantitatively from left to right, as is actually the case. Sachs and Kormann originally made this reaction the basis of a method for quantitative determination of amino groups.[1] Since then a number of other methods based on the same reaction have appeared[2], none of which, however, appears to have satisfied the demands of simplicity, rapidity, and accuracy required to make the reaction available for general use in chemistry and biology.

The method described in the following pages appears to meet these requirements.[3] The complete determination of nitrogen in amino-acids can be finished in a few minutes, and the error kept within ± 0.05 mg. of nitrogen.

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[1] *Zeitschr. f. anal. Chem.*, xiv, p. 380, 1875.

[2] Koenig: *Chem. d. menschl. Nahr. u. Genussmittel,* 4th Edition, iii, p. 274.

[3] The method was first described before the Society of Experimental Biology and Medicine, Dec. 15, 1909, *Proceedings*, vii, p. 46. A preliminary report of the method and its application was published in the *Ber. d. d. chem. Ges.*, xliii, p. 3170, 1910.

PRINCIPLE OF THE METHOD.

Nitrous acid in solution spontaneously decomposes with formation of nitric oxide:

$$2HNO_2 \rightleftarrows HNO_3 + NO.$$

This reaction is utilized in displacing all the air in the apparatus with nitric oxide. The amino solution is then introduced, evolution of nitrogen mixed with nitric oxide resulting. The oxide is absorbed by alkaline permanganate solution, and the pure nitrogen measured in the special gas burette shown in the figure.

REAGENTS.

The permanganate as absorbent for nitric oxide was chosen after trial of all the solutions recommended in the literature. Ferrous sulphate solution, which is ordinarily recommended in gas analysis methods, is entirely unsatisfactory. The reaction by which ferrous sulphate and nitric oxide combine is reversible, and the nitric oxide in solution attains an equilibrium with that in the supernatant gas. Therefor even approximately complete absorption is possible only with perfectly fresh ferrous sulphate solution, and even with this, is a comparatively slow process. Results become inaccurate before the solution has absorbed its own volume of nitric oxide. Sulphite solution, recommended by Divers[1], is even less satisfactory. A strong solution of sodium dichromate in sulphuric acid, which oxidizes the oxide to nitric acid, is better, but is somewhat viscous. Acid permanganate, unless in very dilute solution, gradually decomposes giving off oxygen, which supersaturates the solution. One per cent permanganate in 1 per cent sulphuric acid gives accurate results, however, if the solution is freed from excess oxygen by shaking thoroughly with air immediately before use. Alkaline permanganate, orginally employed by Hans Meyer[2], proved an absolutely satisfactory absorbent solution in every respect. It is entirely stable, can be used in concentrated solution, and oxidizes the nitric oxide to nitrate with such rapidity that the gas is absorbed about as fast as is carbon dioxide by potassium hydrate solution. A solution containing 50 grams of potassium permanganate and 25 grams of potassium hydrate per liter was adopted for permanent use. The manganese dioxide formed by reduction is in such a fine state of division that it does not interfere at all with the use of the solution in a Hempel absorption pipette, and a large number of determinations can be made without changing the solution. In order to prevent deposition of manganese dioxide in the capillaries, it is well to leave *G* (see Fig.) filled with water from the gas burette, rather than with permanganate, when the apparatus is not in use. As the alkaline solution absorbs carbon dioxide as well as nitrogen, the presence of carbonate in the amino solution does not interfere with the determination.

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[1] Classen's *Ausgewählte Methoden*, ii, p. 447, 1903.

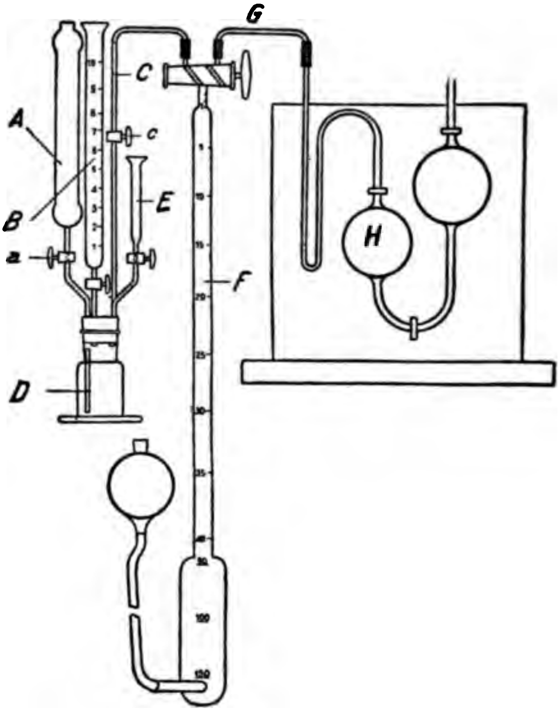
[2] *Analyse und Konstitutionsermittelung*, p. 528, 1903.

For decomposing the amino substance the most satisfactory conditions are, a great excess of nitrite, from which the nitrous acid is freed by an equivalent of a weak acid (acetic). The great excess of reagent forces the reaction to rapid completion. The use of a weak acid, instead of the mineral acids employed in previous methods, causes evolution of a relatively small volume of nitric oxide, and avoids danger of acid hydrolysis of the more complex proteolytic products. In dissolving amino substances not readily soluble in water alone, one may use mineral acids of not more than $\frac{N}{2}$ concentration, acetic acid of any concentration up to 50 per cent, or fixed alkali up to $\frac{N}{1}$ concentration. A few drops of sodium hydrate solution are usually added to assist in dissolving tyrosin and lysin picrate.

Corrections for Impurity in Reagents. As commercial sodium nitrite often contains impurities which gradually evolve traces of nitrogen when the nitrite is acidified, each lot of the latter must be tested before it is used, and, a correction for the reagent employed, if necessary, in calculating subsequent results. A typical "C.P." commercial nitrite yielded 0.2 c.c. of nitrogen in 5 minutes, 0.3 c.c. in one-half hour, and 0.5 c.c. in 2 hours.

APPARATUS.

The apparatus[1] is shown in the figure. The reaction is carried out in *D*, a bottle of 35-37 cc. capacity. It is fitted with a 4-hole rubber stopper, which holds permanently the tubes shown in the figure. The stopper is held firmly in place by a strip of picture wire passing through loops of stout copper wire on opposite sides of the neck of the bottle. All the tubing in the apparatus is capillary, of 6-7 mm. external diameter, and of 1 mm. bore, except the tube from *A*, which is of 2 mm. bore. Cylinder *A*, of 35 cc. capacity, serves to hold water which is used to displace air from *D*, or to receive solution forced back from *D* by nitric oxide. The



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[1] The apparatus is furnished by E. Machlett and Son, 143 E. 23 St., New York City ($12); and by Robert Goetze, Leipsig (Mk. 25).

10 cc. burette *B* holds the solution of amino substance for analysis. Tube *C* serves as an outlet for gases, and connects *D* with the gas burette while the nitrogen is being evolved. The lower end of *C* is exactly flush with the bottom of the stopper. The small cylinder *E*, of 2 cc. capacity, holds amyl alcohol for use in analysis of viscous solutions, such as those containing albumoses, or proteins. The addition of an occasional drop of amyl alcohol prevents foaming of these solutions during the evolution of nitrogen. The gas burette *F* is divided into tenths of a cc. for 40 cc. Below the 40 cc. mark it broadens into a bulb, which is graduated only into 10 cc. divisions. The bulb provides a volume capable of holding the mixture of nitrogen and nitric oxide first liberated, while the finely divided portion of the burette measures the pure nitrogen after the oxide has been absorbed. The water in the gas burette dissolves some of the nitric oxide, which keeps the burette clean by reducing the occasional drops of permanganate carried back with gas from the absorption pipette. Capillary rubber tubing with walls 3 or 4 mm. thick is used to connect *C* and *G* with the gas burette. The absorbent solution in the Hempel pipette is the alkaline permanganate already described.

THE DETERMINATION.

The process may be divided into three stages: (1) Displacement of the air in the apparatus by an atmosphere of pure nitric oxide; (2) Decomposition of the amino substance; (3) Absorption of nitric oxide and measurement of the pure nitrogen. The entire determination usually requires about 10 minutes.

Displacement of Air by Nitric oxide. The solution of amino substance, containing preferably not over 20 mg. of amino nitrogen, is placed in *B*, and 5 cc. of water in *A*. Into *D* one then pours 28 cc. of the solution of sodium nitrite (30 gm. to 100 cc. of water) followed by 7 cc. of glacial acetic acid. Rapid evolution of nitric oxide begins at once. The cock *c* being open, the stopper is now placed in the neck of *D* and fastened firmly with the wire. The small volume of air in *D* is driven out by letting in the water from *A* until the bottle is completely filled and liquid rises in *C*. In order to remove also the air dissolved in the nitrous acid solution, *c* is closed, *a* left open, and *D* is shaken, the tops of *A*, *B*, and *C* being held by the left hand. The shaking causes quick evolution of nitric oxide, which gathers in the top of *D* and forces 10-15 c.c. of solution back into *A*. Cock *c* is now reopened, and the nitric oxide, together with the air which it has swept out of the solution, is forced out of *D* by liquid from *A*. In order to assure complete removal of all traces of air, *c* is closed and the process once repeated. Then, by again closing *c* and shaking *D*, one generates a gas space of about 20 c.c. in *D*, in order to make room for the amino solution from *B*. *G* and *H* being completely filled with permanganate solution, and *F* with the 1 per cent sulphuric up to the top of the rubber connecting tube, *C* and *F* are joined, cock *c* and being opened and *a* closed. The above manipulations require about two minutes.

Decomposition of the Amino Substance. *C* and *F* being connected, the amino solution from *B* is run into *D*, and mixed with the nitrous acid solution. Rapid evolution of nitrogen, mixed with nitric oxide, begins at once. After the reaction has run 5 minutes, in the case of the α-amino acids, or longer, as required for most other amino derivatives (*cf.* pp. 191-192), the evolution of nitrogen is completed by thoroughly shaking *D*.

If proteins, albumoses, or other substances producing viscous solutions are present in the amino solution, a drop of amyl alcohol is occasionally added (from *E*, cf. Fig.) to prevent foaming during the rapid evolution of nitrogen. When, as in digestion experiments, the determination is performed upon proteins or their partially hydrolyzed products, the reaction is run for only 5 minutes, the solution being stirred by shaking several times a minute. Under these conditions there appears to be no danger of decomposition, other than deamination, of the complex substances. The deaminized products, from the proteins and their primary hydrolytic products, are insoluble. Consequently precipitates result from the action of nitrous acid on solutions of proteins undigested or in the earlier stages of digestion. The precipitates do not interfere at all with the determinations. In case ammonia, which does not react as rapidly as primary amino groups, is present, about 15 per cent of it is converted into free nitrogen during the 5-minute reaction at 20°.

Absorption of Nitric Oxide and Measurement of Nitrogen. The reaction being completed, all the gas is driven from *D* and *C* into

F by opening *A* and letting liquid from *A* into *D*. By raising the levelling bulb the gas is driven from *F* into *H*, care being taken that none is left in the connecting capillaries of *G* and the pipette. The nitric oxide is absorbed by shaking the gases with the permanganate solution. The pure nitrogen is run back into *F*, the permanganate filling *G* as far as *f*. The surface of the water in the levelling bulb being brought even with the meniscus, the volume of gas in *F* is measured. The absorption usually occupies about a minute, but varies somewhat with the volume of the nitrogen, the freshness of the permanganate, and the thoroughness of the shaking. It is advisable, until one has a little experience, to test the completeness of the absorption by repeating it, and noting whether the volume of gas is diminished. The room temperature beside the apparatus and the atmospheric pressure are taken, and the weight of nitrogen calculated from the usual tables for nitrogen gas measured over water. As the reaction doubles the amount of nitrogen present in the amino groups, the results are to be divided by 2. Consequently, each milligram of amino nitrogen generates, according to pressure and temperature, 1.7-1.9 c.c. of nitrogen gas, which enables one to obtain very accurate results with relatively small amounts of material. The method is at present in regular use in this laboratory for analytical identification of amino acids.[1]

In the method as above described the only source of error, reagents being pure, is the 0.2 c.c. of air which the 10 c.c. of amino solution can dissolve at atmospheric pressure. As the oxygen combines with the NO to form NO_2, which is absorbed by the permanganate, only the 0.16 c.c. of nitrogen is added to the gas measured. This correction is also indicated by blank experiments. Consequently when the amino solution is saturated with air, 0.16 c.c. is deducted from the nitrogen volume. The correction, which is equivalent to only 0.09 mg. of amino nitrogen, can be avoided by using, in preparation of the amino solution, water which has been freed from air by boiling, or by shaking for a few seconds in an evacuated flask.

Time Required for Different Classes of Amino Derivatives to react Quantitatively. Amino groups in the α-position to carboxyl, as in the natural amino-acids, react quantitatively in 5 minutes at 20°. The group in *lysin* requires one-half hour to react completely, lysin being the only natural amino-acid which requires more than 5 minutes. *Ammonia and methylamine* require 1.5-2 hours to react quantitatively. *Urea* requires 8 hours. In 1 hour it gives off 50 per cent of its nitrogen, and the reaction rate follows the monomolecular equation. Amino groups in *purines* and *pyrimidines* require 2-5 hours at 20°.

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[1] Levene, VanSlyke and Birchard: This *Journal*, viii, p. 269.

In case, for any reason, there is doubt concerning the completeness of the reaction, *C* and *F* are left connected, *a* being open, while the nitric oxide is absorbed and the nitrogen measured. The gas which has meantime collected in the top of *D*, together with that which can be freed from the solution in *D* by shaking, is run over into *F*, freed from nitric oxide, and the nitrogen is again measured. If there is no increase in the nitrogen volume, the reaction was complete at the first measurement.

DETERMINATIONS OF AMINO NITROGEN IN AMINO-ACIDS, PEPTIDES, AND OTHER SUBSTANCES.

The following table contains a series of representative analyses. Most of the amino-acid analyses have since been repeated numerous times in the course of protein hydrolyses. The results with pure leucin illustrate the agreement of duplicates. The results with the other substances are all as close to theoretical as could be guaranteed by the purity of the substances, except glycocoll and cystin. Although they gave theoretical results on combustion, the amino nitrogen always came out about 103 per cent of that theoretically calculated for glycocoll and 107 per cent for cystin. The cause of these errors will be discussed later. The purity of the substances tabulated below was controlled by analyses by the usual methods.

All the amino-acids react quantitatively with their α-amino groups. Lysin reacts with its ω-amino group also, but less rapidly. The guanidin group, in guanidin, creatin, and arginin does not react at all, nor does the nitrogen of the imidazol ring in histidin, the indol ring in tryptophan, or the pyrollidine ring in prolin and oxyprolin. Summarizing the amino-acid results: *every known amino-acid obtained from proteins by acid hydrolysis reacts quantitatively*

TABLE I.

SUBSTANCE	SOURCE	AMOUNT TAKEN	CC. N	TEMPERATURE	PRESSURE	PER CENT N FOUND	PER CENT N CALCULATED	
							1 Atom.	Total organic N
					mm. Hg.			
Leucin	Witte Pepton (Ester method)	0.1311	25.10	22°	754	10.71	10.69	10.69
Leucin..........	Witte Pepton (Ester method)	0.1311	25.00	22°	754	10.66	10.69	10.69
Leucin..........	Witte Pepton (Ester method)	0.1311	25.20	23°	754	10.69	10.69	10.69
Leucin..........	Witte Pepton (Ester method)	0.1232	23.12	21°	766	10.71	10.69	10.69
Leucin..........	Witte Pepton (Ester method)	0.1232	23.05	21°	766	10.68	10.69	10.69
Valin.	Witte Pepton (Ester method)	0.1072	22.10	16°	756	11.89	11.96	11.96
Alanin	Kahlbaum	0.0891	24.50	19.5°	757	15.67	15.73	15.73
Glycocoll	Kahlbaum	0.0732	24.70	20°	752	18.98	18.67	18.67
Tyrosin	Witte Pepton	0.1818	25.20	22°	768	7.89	7.73	7.73
Phenyl alanin...	Kahlbaum	0.1667	25.40	22°	754	8.54	8.49	8.49
Glutaminic acid.	Kahlbaum	0.1457	24.80	20°	756	9.63	9.52	9.52
Aspartic acid...	Kahlbaum	0.1331	25.20	24°	765	10.62	10.54	10.54
Lysin picrate, ..	Witte Pepton	0.1437	18.60	22°	773	7.44}	(7.47)	18.67
Serin...........	Witte Pepton	0.0883	21.10	21°	758	13.50	13.33	13.33
Oxyprolin......	Gelatin					0.00	10.69	10.69
Prolin.........	Gelatin					0.00	12.17	12.17
Histidin dichloride..........	Edestin	0.1636	17.50	22°	762	6.03	6.14	18.42
Tryptophan....	Casein	0.1603	20.80	22°	758	6.94	6.86	13.72
Arginin, $HNO_3.AgNO_3$ salt...........	Edestin	0.2035	12.20	20°	763	3.43	3.44	13.76

TABLE I—CONTINUED.

SUBSTANCE	SOURCE	AMOUNT TAKEN	CC. N	TEMPERATURE	PRESSURE	PER CENT N FOUND	PER CENT N CALCULATED	
							1 Atom.	Total organic N
					mm. *Hg.*			
Guanidin	Merck					0.00		
Creatin..	Merck					0.00		
Asparagin ...... (cryst.)	Kahlbaum	0.1650	27.80	22°	752	9.42	9.34	18.68
Glucosamin chloride..........	Kahlbaum	0.2580	30.40	17°	752	6.73	6.50	6.50
Methylammonium chloride...	Merck	0.0659	24.10	22°	760	20.62	20.75	20.75
Glycin anhydride						0.00		
Glycyl-glycin ...		0.1321	31.0	22°	760	13.13	10.53	26.26
Leucyl-glycin ..		0.0941	13.00	20°	753	7.79		
.		0.0941	13.10	22°	753	7.76	7.45	14.90
Leucyl-leucin ..		0.1307	13.50	21°	760	5.85	5.73	11.46

TABLE II.

Proteins and Intermediate Proteolytic Products.

SUBSTANCE	SOURCE	MG. TOTAL N IN SAMPLE	CC. N	TEMPERATURE	PRESSURE	MG. AMINO N	PER CENT TOTAL N AS AMINO N
Egg albumin..........		16.10	.84	20°	768	.48	2.98
Edestin		30.70	1.40	29°	756	.76	2.47
Hetero-albumose[1]	Witte Peptone	55.34	6.20	23°	772	3.53	6.38
Proto-albumose......	Witte Peptone	39.40	4.40	21°	756	2.48	6.30
Deutero-albumose, B	Witte Peptone	52.64	9.50	22°	760	5.40	10.25
Deutero-albumose, A	Witte Peptone	41.50	9.40	19°	762	5.40	13.01

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[1] Levene, Van Slyke and Birchard; This *Journal*, viii, p. 272.

TABLE III.

Purine and Pyrimidine Ribosides.[1]

SUBSTANCE	SOURCE	AMOUNT TAKEN	CC. N	TEMPERATURE	PRESSURE	PER CENT AMINO N FOUND	CALC.
					mm.		
Cytidin Chloride $C_9H_{13}O_5N_3$. HCl.......	Yeast[1] Nucleic Acid	0.1498	13.00	22°	765	4.93	5.02
Cytidin Nitrate $C_9H_{13}O_5N_3$. HNO_3.....	Yeast Nucleic Acid	0.1149	9.28	19°	758	4.61	4.57
Cytidin Sulphate $(C_9H_{13}O_5N_3)_2.H_2SO_4$...	Yeast Nucleic Acid	0.1568	13.00	21°	772	4.76	4.89
Guanosin I $C_{10}H_{13}N_5O_5.2H_2O$.....	Yeast Nucleic Acid	0.2250	21.40	23°	778	5.46	4.40
Guanosin II $C_{10}H_{13}N_5O_5$...........	Pancreas	0.1344	15.50	23°	764	6.51	4.95
Guanosin III $C_{10}H_{13}N_5O_5$...........	Yeast Nucleic Acid	0.1240	14.30	21°	764	6.57	4.95
Adenosin $C_{10}H_{13}N_5O_4$...........	Yeast Nucleic Acid	0.1607	14.60	19°	770	5.27	5.24

with one and only one nitrogen atom, except lysin, which reacts with two, and prolin and oxyprolin, which do not react at all. All the amino-acids react with all of their nitrogen, except tryptophan, which reacts with one-half, *histidin* with one-third, *arginin* with one-fourth, and *prolin* and *oxyprolin* with none.[2]

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[1] Levene and Jacobs: *Ber. d. deutsch. chem. Ges.*, xliii, p. 3150. Levene and La Forge: *idem*, p. 3164.

[2] Abderhalden's diamino-trioxy-dododecanoic acid was not tested, because of lack of material.

The dipeptids leucyl-leucin and leucyl-glycin react with only their free primary amino groups. The amino nitrogen bound in the -CO-NH- peptid linking does not react. Glycin anhydride, in which both nitrogen atoms are the in imino peptid linkings, gives off no nitrogen at all when treated with nitrous acid.

The proteins, egg albumin and edestin, react, as might be expected, from Fischer's peptid theory of protein structure, with only a trace of their nitrogen, nearly all of the latter being bound in the peptid linkings of the protein molecule. The proportion of free amino groups is twice as great in the primary albumoses, and still greater in the deutero. The smaller the molecule, the greater the proportion of free amino nitrogen, as is already indicated by the results with the peptids.

From the results of Levites[1] and Skraup[2], who found that no lysin could be obtained on hydrolysis of deaminized proteins, it appears probable that a large part of the free amino nitrogen in the native proteins is in the lysin radicle, of which presumably only one of the two amino groups is bound in peptid linking.

Asparagin, as Sachs and Kormann found, reacts only with its α-amino group. It does not react appreciably with the acid-amid nitrogen even when the reaction is prolonged for hours. From this it appears that the conclusions of Schiff[3] are not final. He found that deaminizing proteins with nitrous acid did not remove the "amid" nitrogen, and concludes that this nitrogen can not originate from ($CONH_2$) groups in the protein molecule. As the acid amid nitrogen is not readily decomposed into free nitrogen by nitrous acid, Schiff's results do not prove the point. The work of Osborne, Leavenworth, and Brautlecht[4] makes it very probable that the amid nitrogen does exist in the protein molecule in acid amid combination with the aspartic and glutaminic acid radicals.

The purine and pyrimidine derivatives react normally, except guanosin. Although the purity of this substance was undoubted, as controlled by independent analyses, it regularly yielded about 1½, instead of 1, molecule of nitrogen. Apparently the purine ring is partially broken when nitrous acid acts on guanosin. Guanin itself is so insoluble that it precipitates in the reaction mixture, and only a fraction of it reacts in several hours.

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[1] *Biochem. Zeitschr.*, xx, p. 224, 1909.

[2] *Ann. d. Chem.*, cccli, p. 379, 1906.

[3] *Ber. d. deutsch. chem. Gesellsch.*, xxix, p. 1354, 1896.

[4] *Amer. Journ. of Physiol.*, xxiii, p. 180, 1908.

AMINO-ACIDS WHICH REACT ABNORMALLY WITH NITROUS ACID.

Glycocoll and Glycyl peptids. Glycyl-glycin, unlike the other peptids, reacts not only with its free primary amino nitrogen, but also as Fischer and Koelker have shown,[1] with a part of the secondary nitrogen in the peptid linking. This is doubtless connected with the peculiar behavior of glycocoll itself when treated with nitrous acid. It gives off not only nitrogen, but carbon dioxide and traces of some other gas, which is not absorbed by permanganate, indicating that decompositions deeper than the deamination occur. The behavior of glycocoll and glycyl peptides can be explained in three ways:

(1) The peptid is gradually hydrolyzed by the direct action of nitrous acid, freeing the amino-acids.

(2) The glycollyl radical formed by the deamination is unstable, and decomposes in the reaction mixture.

(3) The *intermediary diazo compound* first formed does not decompose entirely in the normal way, with glycollic acid as the sole product; but a part breaks down by another reaction which completely disintegrates the molecule into carbon dioxide and other products. The disintegration of the radical at the end of the peptid chain breaks the peptid linking, and exposes the nitrogen of the next amino-acid radical.

Only the last explanation is consistent with the facts. The first is refuted by the data of Table I. The peptid linkings of leucyl-leucin, leucyl-glycin, and glycin anhydride are not attacked by nitrous acid. It is evident that the peptid linkings themselves are not hydrolyzed by the reagent, unless one of the acid radicals concerned is destroyed.

The data in the following table show that the second explanation is impossible, and that the third in all probability is correct.

Glycollic acid, even in much larger quantities than could be formed from the amounts of glycocoll analyzed, yields no trace of any decomposition gases. Therefore, the second explanation is

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[1] *Annalen*, cccxl, p. 177.

TABLE IV.

SUBSTANCE	WEIGHT OF SAMPLE	DURATION OF REACTION	NITROGEN	CARBON DIOXIDE	TEMPERATURE	PRESSURE	PER CENT N	PER CENT N CALC. FOR 1 ATOM
		min.	*cc.*	*cc.*		*mm.*		
Glycollic acid.........	0.3060	30	0.00	0.0				
Glycollic acid.........	0.4180	60	0.00	0.0				
Glycocoll...............	0.1110	5	37.2	3.1	19°	766	19.32	18.76
Glycocoll ester-hydrochloride..............	0.1608	5	29.0	1.8	20°	766	10.35	10.05
Glycocoll ester-hydrochloride..............	0.1608	5	29.4	1.0	20°	766	10.49	10.05
Glycyl-glycin..........	0.1321	4	31.0		22°	760	13.13	10.60
Glycyl-glycin..........	0.1388	5	33.8		22°	760	13.72	10.60
Glycyl-glycin..........	0.1343	8	32.9	0.6	19°	760	14.13	10.60
Glycyl-glycin..........	0.1343	60	33.8	0.9	21°	760	14.32	10.60

untenable. Also, the fact that the glycyl-glycin reaction comes practically to an end after 8-10 minutes, when only 0.4 of the secondary nitrogen has been set free, is unexplainable on the basis of a gradual decomposition of the glycollyl radical formed by the initial deamination. Such a process, since the reaction is irreversible, would continue until complete.

The course of the glycyl-glycin reaction is, however, what would be expected in case the diazo compound first formed decomposes in two ways, a portion of the glycyl radical completely disintegrating, while another portion follows the normal reaction course, with formation of stable glycollyl-glycin. The complete disintegration of a portion of the glycocoll-diazo compound explains the origin of carbon dioxide from both glycocoll and glycyl-glycin. It also explains the fact that the reaction with glycyl-glycin takes approximately twice as long for completion as that with the amino-acids. The reaction in this case consists of two deaminations, one following the other, and should therefore cover twice the time of one deamination. The glycollyl radical being stable, the part of the molecule deaminized in the normal manner is not further decomposed, and a portion of the secondary nitrogen (60 per cent in this case) remains stable.

The same reaction probably occurs to a less extent with seryl peptids. When the glycyl group is in the molecule at any place except the end of the chain the peptid reacts only normally, as shown by leucyl-glycin and glycin anhydride.

That traces of a gas other than nitrogen and carbon dioxide are formed from glycocoll, is evident from the fact that all the analyses of glycocoll are somewhat high, even when the carbon dioxide is removed completely by alkaline absorbent. The gas measured is about 103 per cent of the theoretical volume of nitrogen. A considerable number of analyses, other than those tabulated, all gave similar results. If one subtracts 3 per cent of the total amount of nitrogen found from the observed volume, the results are nearly as constant and close to theoretical as in the other amino-acids.

Lysin. Lysin reacts abnormally only in requiring a longer time to react completely than do the other amino-acids. This is due to the fact that lysin reacts with two amino groups, one of which is not in the α position and therefore does not react so rapidly. The rate of reaction is shown by the following figures. For each analysis 0.0888 gram of pure lysin picrate, dissolved in 10 c.c. of very dilute sodium hydrate solution, was used.

TABLE V.

TIME OF REACTION	N GAS	TEMPERATURE	PRESSURE	PER CENT AMINO N	CALC.
min.	*cc.*		*mm.*		
5	9.90	19°	758	6.36	7.47
15	10.75	19°	758	6.87	7.47
15	11.20	20°	758	7.06	7.47
30	11.50	19°	758	7.39	7.47
30	11.80	20°	758	7.54	7.47
50	11.70	19°	758	7.51	7.47

Cystin. The manner in which cystin reacts is shown by the following data.

TABLE VI.

WEIGHT OF SUBSTANCE	TIME OF REACTION	CC. OF GAS OBTAINED AS N	TEMPERATURE	PRESSURE	PER CENT N	PER CENT N CALC.	PER CENT OF CALC. N FOUND
	min.						
0.1152	4	25.30	19°	759	12.52	11.66	107.3
0.1152	30	25.60	19°	759	12.68	11.66	108.7

A slight amount of carbon monoxide was apparently present, but the gas could not be reduced to the theoretical volume by shaking with cuprous chloride solution. The results with cystin are quite constant, however, and by using the factor .926, the method can be utilized in analysis of solutions containing cystin.

The sample of cystin used was obtained by recrystallization from a bladder-stone, and gave the following figures on analysis:

0.1347 gm. substance; 13.5 cc. N (Dumas) at 22.5° C, 758 mm.
0.1061 gm. substance; 0.1157 gm. CO_2; 0.0511 gm. H_2O.
0.0750 gm. substance; 0.1458 gm. Ba SO_4.

	Calculated for $C_6H_{12}N_2S_2O_4$:	Found:
C	29.96	29.75
H	5.03	5.26
N	11.66	11.67
S	26.7	26.7

MEASUREMENT OF THE VELOCITY AND EXTENT OF PROTEOLYSIS BY AMINO NITROGEN DETERMINATIONS.

As Emil Fischer and his pupils have shown, the proteins are to be regarded as chains of amino-acids linked together as in peptids. By hydrolysis the -CO-NH- links are split, with formation of a free $-NH_2$ group from each link. Consequently, in a partially hydrolyzed protien, the *ratio* of the *amino nitrogen already set free to that freed by complete hydrolysis* is a measure of the proportion of the peptid linkings broken, or the extent of the hydrolysis. Also, the *rate* at which the amino groups are freed is the *velocity* of the hydrolysis.

As already shown, the peptid-bound nitrogen, in peptides containing the glycyl group at the end of the chain, can be attacked to some extent by nitrous acid; but few of the known proteins contain enough glycocoll to form such peptides in sufficient amount to appreciably influence the determinations.

Preliminary experiments are entirely in accord with the above deductions from Fischer's theory of protein structure and show that the course of proteolysis can be conveniently followed by amino determinations. Aside from its convenience, this has an advantage over the empirical methods, such as tannic acid precipitation, salting out, viscosity measurements, etc., used in the study of proteolysis, in that it permits a direct chemical interpretation of the results: it shows the proportion of peptid linkings broken. The extent of hydrolysis is calculated from the equation:

$$\text{Per cent of hydrolysis} = \frac{100\,(A - A_0)}{A_1 - A_0}$$

A signifies the observed amino nitrogen; A_0 the amino nitrogen of the intact protein before hydrolysis; A_1, the amino nitrogen after complete hydrolysis.[1]

TABLE VII.

Digestion of Edestin by Trypsin.

150 cc. H_2O; 6 gm. air dried edestin; 0.5 gm. Na_2CO_3; 0.6 gm. Grübler's trypsin. Temperature 37°. Portions of 5 cc. removed at intervals for determination of amino nitrogen.

HOURS	CC. N GAS REDUCED TO 0°, 760 MM.	PER CENT OF THE N	PER CENT HYDROLYSIS
0	1.97*	3.68*	0.00
2	7.62	14.93	14.77
4	8.92	17.47	18.15
20	12.62	24.75	27.40
80	19.56	38.35	47.30
Complete hydrolysis by HCl....	40.25	79.00	100.00

*0.77 cc. of the nitrogen, or 1.5 per cent, is due to amino nitrogen introduced with the trypsin. Of the edestin itself, only 2.4 per cent of the nitrogen reacts with nitrous acid.

Hydrolysis of Egg Albumin by Na OH

100 cc. H_2O; 2 gm. air-dried albumin; 5 gm. NaOH. Temp. 60°. Portions of 5 cc. for amino nitrogen determinations.

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[1] As A_0 is relatively small, it can be left out of the formula when conditions prevent experimental determination of its value, as when the undigested protein is insoluble, and approximate results can be obtained by the equation: $\text{hydrolysis} = \frac{100\,A}{A_1}$

HOURS	CC. N GAS REDUCED TO 0°, 760 MM.	PER CENT OF THE TOTAL N	PER CENT HYDROLYSIS
0	0.78	2.85	0.00
0.5	1.85	7.15	5.19
4.5	5.04	19.45	19.95
25	10.11	39.02	43.70
48	12.09	46.62	53.02
96	15.85	61.10	70.70
144	17.75	68.42	83.20
Complete hydrolysis by HCl....	22.10	85.20	100.00

For complete hydrolysis portions were boiled 16 hours with 20 per cent hydrochloric acid. The free acid was removed as far as possible by concentration on the steam bath; the residues were taken up in water and used for duplicate amino and Kjeldahl determinations.

USES OF THE AMINO DETERMINATION.

In conclusion we summarize the uses to which the amino determination can be put.

1. *Measurement of the velocity and extent of proteolysis.* This has been described in the immediately preceding paragraphs. As corollaries we have:

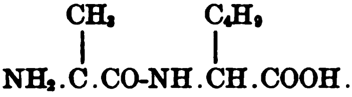
(a) *Determination of the relative digestibility of proteins.* Because of the ease with which the course of hydrolysis can be followed, the amino method will afford a convenient means for determining the relative rates at which different proteins are hydrolyzed by enzymes, acids, or alkali.

(b) *Quantitative determination of proteolytic enzymes.* With a given protein or peptid, the rate at which the peptid linkings are broken is a function of the active enzyme, and can consequently be used to determine the latter. The problem of ascertaining the details of a practical method based on this principle will be taken up as soon as possible.

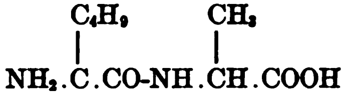
II. *Analysis of amino-acids.* As shown in table I, the nitrogen determinations by the nitrous acid method are fully as accurate as those by the Kjeldahl and Dumas methods. Because of its quickness and simplicity, this means of analysis has proven extremely convenient in identifying and testing the purity of the amino-acids obtained in hydrolysis of proteins.

III. *Determination of the complexity and structure of peptids and proteolytic products.* A polypeptid of monoamino-acids contains only one free NH_2 group in the molecule, that at one end of the chain. Complete hydrolysis frees all the amino groups, so that one is present for each amino-acid. Consequently the ratio (amino nitrogen after hydrolysis): (amino nitrogen of intact peptid) expresses the number of amino-acids combined to form the peptid. Prolin and oxyprolin, which contain no amino groups, and lysin, which contains two, are special cases and would not fall within this rule, but all of the other amino-acids react as monoamino-acids. Consequently the nitrous acid reaction will serve to estimate approximately the average size of the peptids in the mixture from a partially hydrolyzed protein, and should be of material assistance in determining the molecular size and structure of individual peptids isolated from such mixtures. The results in Table I with proteins, primary and secondary albumoses, and peptids afford experimental basis for the above statements.

As an amino-acid radical, so situated at one end of the peptid chain that its amino group is free, its carboxyl bound in a (CO-NH) linking, is changed to an α-hydroxy acid radical by the action of nitrous acid, the isolation of an α-hydroxy acid after first deaminizing, then hydrolyzing a peptid, indicates the position, in the peptid, of that amino-acid from which the hydroxy acid is derived. For example, if a dipeptid should yield alanin and leucin on direct hydrolysis, but lactic acid and leucin on hydrolysis after previous action of nitrous acid, the peptid would be alanyl-leucin,



$NH_2.\overset{\overset{CH_3}{|}}{C}.CO\text{-}NH.\overset{\overset{C_4H_9}{|}}{CH}.COOH$. If the products of deamination and hydrolysis were alanin and α-hydroxy-isocaproic acid the peptid leucyl-alanin,



$NH_2.\overset{\overset{C_4H_9}{|}}{C}.CO\text{-}NH.\overset{\overset{CH_3}{|}}{CH}.COOH$ would be indicated. Glycyl peptids, as before pointed out, react abnormally.

IV. *Characterization of proteins.* The amino-acids derived from proteins may be divided into two groups, those which react with nitrous acid with all of their nitrogen, and those which react with none or only a fraction of their nitrogen. The latter group consists of prolin, oxyprolin, arginin, histidin, and tryptophan. The proportions in which these two groups are present in a protein can be readily and accurately determined by hydrolyzing, removing the ammonia by aëration[1] or boiling *in vacuo* with lime, and determining total and amino nitrogen in the solution. The ratio of amino to non-amino nitrogen thus obtained is probably the most characteristic and accurately determinable general chemical constant of the proteins as a class. It should prove not less useful than the nitrogen distribution method of Hausmann, which, as developed by Osborne and Harris, divides the acids into two groups, the "bases," which are precipitated by phosphotungstic acid, and the other amino-acids, which are not. By a combination of the phosphotungstic precipitation with amino determination and special methods for arginin and cystin it is possible to determine the different hexone bases and obtain a fairly complete picture of the proportion in which the different types of amino-acids enter into the composition of a protein, using for the purpose only two or three grams of material. This method and results obtained with it will form the subject of a future paper.[2]

V. *Determination of Amino Nitrogen in Urine.* A preliminary description of this has already been published.[3] The work in full will be published in this journal.

VI. *Quantitative Determination of the Prolin obtained by the Ester Method of Protein Hydrolysis.* The determination of prolin in casein, with the aid of the amino determination, is described in the paper following.

Addendum. When a large number of amino determinations are to be made by the method described in this paper, it is of advantage to use two of the 35 cc. bottles (cf. p. 188), each fitted with stopper, 10 cc. burette etc. While one determination is being carried out, the next can be carried through the first stage; thus six ordinary (α-amino) determinations may be performed in an hour.

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[1] Denis: This *Journal*, v, p. 427.

[2] A preliminary description has been published in the *Proceedings of the Society for Experimental Biology and Medicine*, Report of Meeting, May 18, 1910.

[3] *Proc. Soc. for Exp. Biol. and Med.*, vii, p. 48, Dec. 15, 1909.

QUANTITATIVE DETERMINATION OF PROLIN OBTAINED BY THE ESTER METHOD IN PROTEIN HYDROLYSIS. PROLIN CONTENT OF CASEIN.

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(Received for publication, March 6, 1911.)

Prolin is ordinarily determined in the ester hydrolysis by alcoholic extraction of the amino-acids whose esters boil below 90° at less than 1 mm. pressure. If the amino-acids soluble in absolute alcohol are all calculated as prolin, however, the results are too high; for the prolin is always accompanied by portions of the other amino-acids, from which it cannot be separated by alcohol. If, on the other hand, the impure prolin is racemicized and recrystallized as d-l-copper salt, only a portion of that present can be separated from the mixture, because of the tendency of the other copper salts to crystallize with it.

The prolin content can be readily and accurately ascertained by determination of the total and amino nitrogen of the alcohol-soluble mixture. Each of the amino-acids whose esters distil with that of prolin gives off all its nitrogen when treated with nitrous acid in the amino determination described in the foregoing paper. Prolin, on the contrary, does not react at all. Consequently one can ascertain the prolin content of the mixture by subtracting the amino nitrogen, the difference being prolin nitrogen.

464 grams of casein were hydrolyzed, esterified by Fischer's method, and the esters freed three times with barium hydrate as described by Levene and Van Slyke.[1] The total yield of distilled esters was 374 grams. The amino-acids yielded by the esters boiling below 90° at 0.5 mm. pressure were extracted with abso-

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[1] This *Journal*, vi, p. 419, 1909.

lute alcohol. The alcoholic solution was concentrated as far as possible on the water bath, then taken up with cold absolute alcohol, and filtered. Repetition of this process gave a product completely soluble in cold absolute alcohol. It was freed from alcohol by concentrating, taken up in water, and Kjeldahl and amino determinations were performed upon aliquot parts of the solution. The results were:

	Grams
Total nitrogen	5.441
Amino nitrogen	1.666
Prolin nitrogen	3.775

The 3.775 grams of prolin nitrogen correspond to 31.10 grams of prolin, which is 6.70 *per cent of the casein.*

The amino-acids in a portion of the solution which contained 3.44 grams of nitrogen were racemicized by heating in the autoclave with baryta, then transformed into copper salts. The yield was 38.0 grams of anhydrous salts. Recrystallizing from water yielded 19.0 grams of nearly pure d-l-prolin copper salt, corresponding to 61.5 per cent of the prolin present as calculated from the nitrogen analyses, in the portion racemicized. The substance gave the following figures on analysis:

0.3824 gm. subst.; 0.0416 gm. loss at 100° *in vacuo.*
0.3561 gm. subst.; 10.78 cc. $\frac{N}{10}$ sulphocyanate, Volhard copper titration.
0.3634 gm. subst.; 2.40 cc. nitrogen at 22°, 760 mm. (nitrous acid method).

	Calculated for $Cu(C_5H_8O_2N)_2.2H_2O$:	Found:
H_2O	11.00	10.88
Cu	19.40	19.27
Amino N	0.00	0.34

Calculating the impurity in the form of other amino acids from the amino nitrogen, the substance contained 4.4 per cent of their salts, 95.6 per cent of pure prolin salt.

From the mother liquors a second crop of 3 grams of anhydrous copper salt was obtained by crystallization, and a third crop of 18 grams by evaporating to dryness the filtrate from the second. In the anhydrous salts could be seen granules of the violet prolin copper salt mixed with the blue salts of the other amino-acids. The mixture gave the following figures on analysis.

	CROP 2	CROP 3	CALCULATED: Cu SALTS OF		
			PROLIN	VALIN	ALANIN
N	9.53	9.97	9.60	9.44	11.69
Amino N	4.40	5.66	0.00	9.44	11.69
Cu	21.70	22.43	21.80	21.50	26.53
C	.....	33.81	41.14	40.57	30.03
H	.....	5.69	5.53	6.83	5.05

Separation of more prolin by crystallization of the copper salts was impossible. Later work has shown that from such mixtures as the second and third crops described above it is possible to isolate a portion of the prolin by decomposing the copper salts with hydrogen sulphide and extracting the regenerated amino-acids again with alcohol. A portion of the valin and alanin remains insoluble, and from the alcohol-soluble portion a considerable part of the prolin can be isolated as copper salt. For estimation of the amount of prolin present, however, the determination of the total and amino nitrogen contents of the mixture appears to be not only the simplest method, but the only one available which is quantitative.

The amino determination is also the most delicate test for the purity of prolin isolated from mixtures of amino-acids. The other acids may be present in considerable amounts without noticeably affecting the elementary composition of the prolin. Valin, for example, gives nearly the same analytical figures. The presence of even traces of the straight-chain amino-acids, however, is revealed, both qualitatively and quantitatively, by the nitrous acid reaction.

The prolin content of casein, found as described above, is twice that found by Abderhalden,[1] but agrees with that found by Engeland with his methylation method.[2]

[1] *Zeitschr. f. physiol. Chem.*, xlvi, p. 23.

[2] *Ber. d.d. chem. Gesellsch.*, xlii, p. 2962.

DIGESTION OF PROTEIN IN THE STOMACH AND INTESTINE OF THE DOGFISH.

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Experiments on the course of digestion and absorption of protein in different parts of the alimentary canal of the dog have been performed by Schmidt-Mülheim,[1] and later by London and Sivre,[2] Abderhalden, Medigreceanu, and London,[3] and Abderhalden, L. Baumann and London.[4] Similar work on the cold-blooded vertebrates has been lacking, however. The present work purposed to obtain data in this field, and also to follow the course of the protein cleavage by means of the nitrous acid determination of primary amino nitrogen, which affords a direct chemical measure of the extent and rate of protein hydrolysis.[5]

METHODS.

Beef, which had been chopped and boiled to coagulate the proteins, was fed to the fish through a short stomach tube. Specimens of as nearly uniform size as possible were used, and the amount of meat fed was graduated according to the square of the animals' length, in accordance with Rubner's law of energy requirement. The fish were killed after 6, 12, 24, 48, or 72 hours. The intestine was ligated near the pylorus and cloaca and the stomach at the pylorus and œsophogus in order to avoid loss of contents. Both organs were then removed, and the contents washed out. The mixtures obtained were centrifugated to separate the solids, and the supernatant

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[1] *Arch. f. Physiol.*, 1879, p. 29.

[2] *Zeitschr. f. physiol. Chem.*, lx, pp. 191, 194.

[3] *Ibid.*, lviii, p. 435.

[4] *Ibid.*, xlviii, p. 549, 1907.

[5] Van Slyke: This *Journal*, ix, p. 185, 1911.

liquid was filtered through glass-wool to remove a few particles usually remaining in suspension. The solids were washed, also by centrifugation, and the nitrogen in the insoluble residue was determined by the Kjeldahl method.

The washings were united, brought to a convenient volume, 50 to 200 cc., and aliquot parts used for determination of the total nitrogen, and of the primary amino nitrogen by the nitrous acid method. Another portion of the solution was hydrolyzed by two days' boiling under a reflux condenser with 20 per cent hydrochloric acid. The acid was then driven off as completely as possible by concentrating the solution to a syrup on a water bath. The residue was diluted to a definite volume, and used for determination of the amino nitrogen in the completely hydrolyzed contents. The ratio of (amino nitrogen after hydrolysis): (amino nitrogen before hydrolysis) indicates the average size of the peptides in the digesting solution. The urea determinations were made by the method of Levene and Meyer.[1]

The following results in detail, from the stomach contents in experiment No. 5 serve as an illustration.

The fish received 40 grams of beef containing 5.05 per cent nitrogen, and was killed 12 hours later.

The soluble stomach contents obtained after centrifugating twice were diluted to 150 cc. and analyzed.

Total Nitrogen, 10 cc. samples, 21.03–21.05 cc. $\frac{N}{10}$ H_2SO_4.
Nitrogen in 150 cc. = 0.442 gram.

Amino Nitrogen. 10 cc. sample, 15.00 cc. of nitrogen freed by nitrous acid, at 25°, 760 mm. Amino nitrogen in 150 cc. = 0.125 gm.

Amino Nitrogen after Hydrolysis. Twenty-five cc. were mixed with 25 cc. of concentrated hydrochloric acid and boiled 16 hours. The solution was concentrated, then diluted to 50 cc. 10 cc. gave 19.50 cc. of nitrogen at 25°, 760 mm., equivalent to 0.325 gm. of amino nitrogen in the original 150 cc.

Washings. The washings, after the first, were analyzed separately for total nitrogen, in order to avoid inconvenient dilution of the portion used for amino determinations. The washings contained 0.324 gram of nitrogen. Consequently the total soluble nitrogen was $0.442+0.234=0.676$ gm. To apply to the total soluble nitrogen, the amino results therefore are multiplied by $\frac{676}{442}$.

Solids. These, by the Kjeldahl method, neutralized 28.02 cc. of normal acid, equivalent to 0.392 gm. of nitrogen.

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[1] *Journ. Amer. Chem. Soc.,* xxxi, p. 717, 1909.

EXCRETION OF UREA BY THE LIVER.

In performing the amino nitrogen determinations upon the intestinal contents two facts were regularly noted which indicated that a considerable portion of the soluble nitrogen did not originate from proteins or their hydrolytic products. First the amino nitrogen after complete hydrolysis amounted to only 40-50 per cent of the total soluble nitrogen. Hydrolyzed beef protein contains 75 per cent of its nitrogen in amino form. Second, when nitrous acid was allowed to react for a long time, 5 hours instead of 5 minutes, the amount of nitrogen freed was increased. The increase was sufficient, after hydrolysis, to raise the amino nitrogen to 70-80 per cent of the total. From the above behavior it appeared that 20-40 per cent of the soluble nitrogen was in a form decomposed by nitrous acid, but reacting slowly instead of in 3-5 minutes like the amino-acids and peptides. Urea and ammonia react in this manner. Ammonia determinations by the Folin aëration method showed only a few per cent of the nitrogen in this form. Urea determinations were then performed upon the soluble portion of the contents, by the method of Levene and Meyer, with the results that 25-58 per cent of the total soluble nitrogen was found in the form of urea (cf. Nos. 10, 11 and 15, Table II.)

The probable source of this was either the bile, or the urine which might conceivably diffuse back from the cloaca into the intestine. In order to determine whether this occurred, the gut was thoroughly flushed out from the pylorous down with a stream of water before the fish were fed. The flushing is easily performed from the anus with a glass tube, because the intestine is straight. The abdomen was then opened above the pelvic girdle and the intestine firmly ligated near the cloaca. The wound was sutured and the fish fed. The operation appeared to inconvenience the animals only in causing a reversal of the peristaltic waves, resulting in regurgitation of part of the food. After 48 hours the fish were killed and the alimentary contents examined as usual. The intestines contained about the same proportion of urea nitrogen as those of normal fish, and the amino determinations ran the same course.

The urine being excluded, the bile appeared the probable source of the urea. From the gall bladders of several fish 4 cc. of bile was obtained. This was diluted to 25 cc. and 5 cc. used for a Kjeldahl determination. 6.29 cc. of $\frac{N}{10}$ acid were neutralized, indicating 0.04403 gm. of nitrogen in the 25 cc. For urea determination 10 cc. were taken, and required 9.10 cc. of acid, indicating 0.0318 gm. of urea nitrogen in all, or urea itself equal to 1.7 per cent of the bile. The urea nitrogen constituted 72.3 per cent of all the nitrogen in the bile. It is evident therefore that the bile duct constitutes an important avenue for the excretion of urea, and is the source of the urea found in the intestinal contents.

The presence of urea in dog-fish bile has already been qualitatively proved by Hammarsten, who crystallized and identified the substance, but did not determine the proportions in which it was present.

The behavior of the stomach contents indicated the presence of but little nitrogen other than that derived from the food proteins. The amino nitrogen after total hydrolysis ran from 65 to 75 per cent of the total. Apparently urea is not excreted into the alimentary canal through its walls but only through the bile duct.

The figures concerning the protein digestion are given in Tables I and II. The figures for amino-acid nitrogen in the soluble intestinal contents are not absolutely accurate, because of the presence of the urea. The amino determinations were uniformly made in 5 minutes, however, and urea reacts so slowly with nitrous acid under the conditions of the determination that the error does not affect materially the significance of the results.

As mentioned, experiments 14, 15 and 16 were performed on animals with ligated intestines. Part of the food was regurgitated by each of these animals, so the results cannot be quantitatively compared with those from normal fish.

NO.	LENGTH	HOURS BETWE FEEDING AND KILLING	GRAMS N FED	GRAMS IN ALIM ARY CANAL	TOTAL N	INSOLUBLE N	SOLUBLE N	SOLUBLE AMIN	SOLUBLE AMIN AFTER HYDR SIS	TOTAL N	INSOLUBLE N	SOLUBLE N	SOLUBLE AMIN	SOLUBLE AMIN AFTER HYDR SIS	UREA
	(cm.)														
1	65	6	1.29	0.962	0.872	0.577	0.295	0.036	0.180	0.090	0.025	0.065	0.007	0.019	
2	70	6	1.50	0.824	0.779	0.374	0.405	0.054	0.282	0.045	0.012	0.032	0.005	0.012	
3	65	12	1.35	0.978	0.690	0.292	0.398	0.087	0.264	0.288	0.112	0.176	0.036	0.079	
4	75	12	2.02	1.939	1.056	0.603	0.453	0.085	0.276	0.883	0.638	0.245	0.046	0.130	
5	75	12	2.02	1.530	1.068	0.392	0.676	0.191	0.497	0.462	0.169	0.293	0.099	0.171	
6	50	24	0.99	0.288	0.153	0.052	0.101	0.029	0.070	0.135	0.021	0.114	0.024	0.0563	
7	70	24	1.79	0.770	0.626	0.171	0.455	0.118	0.316	0.144	0.032	0.112	0.027	0.053	
8	83	24	2.02	1.172	0.800	0.165	0.635	0.168	0.410	0.327	0.125	0.247	0.037	0.102	
9	65	48	1.44	0.640	0.366	0.071	0.295	0.086	0.194	0.274	0.115	0.159	0.033	0.079	
10	75	48	1.72	0.239	0.135	0.059	0.076	0.017	0.047	0.104	0.025	0.078	0.011		0.028
11	75	48	1.72	0.240	0.072	0.017	0.055	0.010	0.027	0.168	0.066	0.102	0.019	0.034	0.060
12	82	72	1.98	0.069	0.023	0.000	0.023			0.046	0.000	0.046	0.003		
13	70	72	2.50	0.262	0.138	0.011	0.127	0.032	0.079	0.124	0.014	0.110	0.023	0.042	
Specimens With Ligated Intestine															
14	80	24	1.93	0.443	0.397	0.109	.288	0.072	0.171	0.046	0.012	0.034	0.004	0.014	
15	85	24	2.19	0.131	0.100	0.009	.091	0.016	0.041	0.031	0.000	0.031	0.004		0.0116
16	76	24	1.72	0.164	0.015	0.001	.014	0.004	0.017	0.149	0.070	0.079	0.0104		0.0160

TABLE II.

NO.	HOURS BETWEEN FEEDING AND KILLING	PER CENT OF FED N IN ALIMENTARY CANAL	STOMACH PER CENT OF ALIMENTARY CANAL N IN STOMACH	STOMACH PER CENT OF STOMACH N IN SOLUTION	STOMACH PER CENT OF SOLUBLE N IN AMINO FORM	STOMACH AMINO N AFTER HYDROLYSIS / AMINO N BEFORE HYDROLYSIS	INTESTINE PER CENT OF ALIMENTARY CANAL N IN INTESTINE	INTESTINE PER CENT OF INTESTINE N IN SOLUTION	INTESTINE PER CENT OF SOLUBLE N IN AMINO FORM	INTESTINE AMINO N AFTER HYDROLYSIS / AMINO N BEFORE HYDROLYSIS	INTESTINE PER CENT OF SOLUBLE N IN FORM OF UREA
1	6	74.6	90.6	33.8	12.2	5.00	9.4	72.2	10.3	2.82	
2	6	54.9*	94.6	52.0	13.3	5.22	5.4	71.1	15.0	2.42	
3	12	72.4	70.6	57.7	21.8	3.03	29.4	61.1	20.4	2.21	
4	12	96.0	54.5	42.9	18.8	3.25	45.5	27.8	18.6	2.85	
5	12	75.7	69.8	63.3	28.3	2.60	30.2	63.4	33.8	1.72	
6	24	29.1	53.1	66.0	28.7	2.41	46.9	84.5	20.7	2.39	
7	24	43.0	81.3	72.7	25.9	2.68	18.7	77.8	23.8	1.98	
8	24	58.0	68.3	79.4	26.5	2.45	31.7	66.4	14.8	2.78	
9	48	44.4	57.2	80.6	29.1	2.25	42.8	58.1	20.7	2.49	
10	48	13.9	56.5	56.3	22.4	2.76	43.5	75.0	12.8		25.7
11	48	14.0	30.0	76.4	18.2	2.70	70.0	60.7	18.9	1.76	58.6
12	72	3.5	33.3	100.0			66.7	100.0	6.7		
13	72	10.5	52.7	92.0	25.2	2.47	47.3	88.7	20.5	1.87	
Specimens with Ligated Intestine.											
14*	24	23.0*	89.7	72.6	25.0	2.38	10.3	74.0	11.2	3.66	

RESULTS.

Between two and three days were required for complete disposal of the meal.

The course of digestion varied considerably with different individuals, but the following facts are shown by the figures.

During the first 6 hours a considerable part of the coagulated protein in the stomach is dissolved and absorbed. Little or none appears to pass into the intestines, which contain little more nitrogenous matter than the intestines of starving dog-fish. In the case of No. 1, 44.7 per cent of the ingested nitrogen remained undissolved in the stomach, 22.9 per cent remained in the stomach in solution, and 7 per cent was found in the intestine, leaving at least 25 per cent which had probably been absorbed from the stomach. As the intestinal nitrogen undoubtedly did not all come from the meal, probably a higher figure than 25 per cent represents the correct extent of absorption during the first 6 hours. The passage of stomach contents into the intestine had hardly begun, and removal of any of the ingested nitrogen by defecation was hardly possible. The average size of the peptids composing the peptone in the stomach at the end of the 6 hour period was that of a pentapeptid. The amino nitrogen content is a little higher than that found in the deutero albumoses from Witte peptone.[1] Evidently the protein is hydrolyzed rapidly after it gets into solution.

During the 6-12 hour period the most noticeable changes are the passage of protein, both digested and solid, into the intestine, and the progressive hydrolysis of the dissolved peptone. The intestine now holds 30-45 per cent of the total nitrogen in the alimentary canal. The peptone in the stomach is broken down on an average to the tripeptid stage, that of the intestine a little farther.

By the end of 24 hours 40-70 per cent of the nitrogen has disappeared, and of that left, in both stomach and intestine, 65-85 per cent is in solution. The average complexity of the peptone in the stomach has fallen to about midway between the di- and tripeptid stage. Further cleavage apparently does not occur in gastric digestion.

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[1] This *Journal*, ix, p. 194, 1911.

During the second 24 hours the disappearance of both soluble and insoluble protein proceeds, in 2 cases out of 3 only 14 per cent of the ingested nitrogen being left in the digestive tract. The cleavage of the unabsorbed peptone, however, goes no farther than in the first 24 hours.

By the end of the third day, solution and absorption of the protein is practically complete in one case; in the other only 10 per cent of the ingested nitrogen, nearly all dissolved, remains in the tract. The degree of cleavage of the peptone is practically the same as that attained after 24 hours.

Throughout the digestion the soluble proteolytic products in the intestine show practically the same degree of cleavage, which does not differ greatly from that attained in the stomach after 24 hours.

Peptonization of the protein in the stomach frees the accompanying fat, which collects in a layer when the stomach contents are centrifugated. Lipolysis occurs rapidly in the intestines; for no fat layer could be detected in any sample of intestinal contents.

Urea is regularly present in the intestinal contents. Animals in which the intestine was flushed out and ligated 24 hours before killing still showed a normal proportion of urea in their intestinal contents. This excludes the possibility that the urea could be due to urine diffused back from the cloaca. The urea comes from the bile, which, in the samples analyzed, contained 72 per cent of its nitrogen in the form of urea. Evidently in the shark family the liver shares with the kidney the function of excreting urea.

COMPARISON WITH DIGESTION IN MAMMALS.

The most noticeable difference in digestion between the warm-blooded and cold-blooded carnivora is in the time required for digestion and absorption. Schmidt-Mülheim found that meat was digested completely and absorbed to the extent of 95 per cent in 12 hours. About six times as long is required in the dogfish. London and Sivre[1] found that in a dog, which was fed meat, half of the nitrogen had moved into the duodenum in 1 hour, and in 5 hours the stomach was empty. In the dogfish the stomach is not entirely empty after 48 hours. The relative slowness with which digestion proceeds in the fish is doubtless partly due to the slower rate of chemical activity at the lower temperature. As there is about 20 degrees difference between the temperature of dog and the fish, the same chemical reactions, according to the van't Hoff law, would proceed 4 to 9 times as fast at the higher temperature, a difference of about the order of magnitude found.

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[1] *Loc. cit.*

In both the fish and mammals, transfer of chyme to the intestine occurs after the proteins in the stomach have been only partially peptonized. The intestine receives some protein which has not even been dissolved by the gastric juice.

Regarding the relative completeness with which the digestive juices of the dog and dogfish respectively hydrolyze the ingested proteins, a satisfactory comparison can be made only after the gastric and intestinal contents of dogs have been examined with the amino determination method. The results of Abderhalden, Medigreceanu, and London, and of Abderhalden, Baumann, and London show that at no point in the alimentary canal of the dog is protein all broken down to amino-acids, a large proportion of the nitrogen being precipitable with phosphotungstic acid, and not obtained with the Fischer method for amino-acid esters. It appears probable that cleavage proceeds as far in the canal of the fish as in that of the dog. That greater variation in the contents of different sections of the dog's tract occurs, is natural, considering the much greater complexity of the dog's intestine.

The presence of large amounts of urea in the bile, as in the blood, appears peculiar to the shark family.

In future investigations of this nature, information of value can doubtless be obtained by determining the proportion of amino nitrogen, both before and after complete hydrolysis, separately, in the portion of the alimentery contents precipitable by phosphotungstic acid, and in the fraction not precipitable.

We wish to thank Prof. G. H. Parker, of Cambridge, and Prof. G. G. Scott, of New York, for friendly advice and assistance in operations on animals used in some of the experiments above reported.

THE RELATION BETWEEN THE DIGESTIBILITY AND THE RETENTION OF INGESTED PROTEINS.

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THE RELATIONS BETWEEN DIGESTION AND ASSIMILATION.[1]

The rate of the catabolism of a protein is determined principally by two factors, namely by the rate of its digestion and by that of its absorption. In the process of protein assimilation, according to the prevailing views, the controlling influence is exercised by the same two factors. Many writers assume that the reconstruction of body protein from the ingested material begins only after its complete deterioration. On the basis of these theories the value of a protein for the organism rises with the increase in its digestibility.

However, there are recorded facts which are not in full harmony with this assumption. Thus Falta first demonstrated that the degree of nitrogen retention is determined by the nature of the ingested proteins. This view was corroborated by Graffenberger and very recently by Voit. According to Falta the fragments of protein molecule which resist the destructive influence of the gastro-intestinal juices are those that are retained the longest in the organism, and are, perhaps, utilized for the purpose of protein assimilation. In a recent series of articles Levin, Manson and Levene, and Carrel, Meyer and Levene have reported experiments of an entirely different character which demonstrate the correctness of this view.

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[1] The problem was suggested by Dr. P. A. Levene. Preliminary work was begun at Woods Hole in the summer of 1909 with Dr. Wm. M. Clark, at present of the U. S. Dept. of Agriculture, and C. B. Bennett of the University of Vermont.

It is known that the act of digestion and absorption is accomplished principally in the intestinal tract, while in the stomach the rate of protein absorption is low and the digestion does not pass the stage of proteoses.

On the basis of the generally accepted view of protein metabolism it seems reasonable to expect that the factors facilitating the transportation of a protein from the stomach to the intestinal tract should increase also the value of protein foodstuffs. The observations of Levene and his co-workers are contradictory to this expectation. They brought to light the fact that, after gastrectomy or gastro-enterostomy, when the food enters directly into the intestinal tract, the rate of nitrogen elimination is high, but the rate of retention is very significant; while the results are reversed after resection of most of the small intestine. On the basis of these experiments it seemed more reasonable to expect that factors causing a delay in transportation of protein foodstuff from the stomach to the intestinal tract would result in a higher rate of nitrogen retention.

The present investigation was originally undertaken with the purpose of establishing the food value of the proteins of the fish meats compared with each other and with that of lean beef. In course of the experiments it was found that the meats varied markedly, according to their source and mode of preparations, in the readiness with which they were digested. It was found possible, therefore, to test the influence of variations in the digestibility of proteins, shown by the curves of nitrogen elimination after their administration, upon the degree of nitrogen retention. The results are in harmony with the views expressed by Falta and by Levene and his co-workers.

THE NITROGEN EXCRETION CURVE AFTER PROTEIN FEEDING.

The curve for the excretion of nitrogen in the urine after protein feeding has been studied by a number of experimenters, beginning with Becher in 1855 and Voit in 1857. The literature is reviewed in recent papers by Stauber[1] and by Haas[2]. The results

[1] Stauber: *Biochem. Zeitschr.*, xxv, p. 187, 1910.

[2] Haas: *Ibid.*, xii, p. 203, 1908.

may be summarized briefly. In the case of a man or dog on a normal diet the greater part of the protein nitrogen consumed at a meal is rapidly transformed in the body and excreted in the urine. Consequently the hourly nitrogen excretion rises rapidly after a meal, and falls again to the original height, which may be termed for convenience the "fasting level," after digestion and absorption are concluded. The nature of the excretion curve is not markedly affected by bodily rest or activity (Haas), but is dependent upon the nature and amount of protein ingested, and, in man at least, to some extent upon the water intake. When larger amounts of protein are fed, more time is required for the curve to return to the fasting level. The nitrogen of certain proteins, as found by Falta[1], and Graffenberger[2] is metabolized more slowly than that of others.

In man, the excretion curve during digestion may be irregular. Within a short time after eating (half an hour, Veraguth[3]), a minor maximum usually occurs, due apparently to diuresis following the intake of water, which is quickly excreted, and washes out the nitrogenous waste products from the tissues (Haas). A second and third maximum usually occur 2 to 4 and 4 to 7 hours after a meal following, Tschlenoff[4] suggests, the periods of greatest resorptive activity in the stomach and intestine respectively. Stauber[5] found the highest excretion normally about 5 hours after feeding. When meat, predigested by pepsin was eaten, however, the chief maximum occurred within 1–2 hours, indicating that the products of protein digestion are absorbed and excreted soon after they are formed.

In experiments with dogs, in which the animals were fed meat equal to 2–4 per cent of the body weight and catheterized at intervals, the bladder being washed out thoroughly, the curve of nitrogen excretion following a meal was found to rise regularly to a maximum, which occurs after 4–8 hours, then to fall gradually to the fasting level, reached after about 20 hours.[6] Comparison with the rate of absorption from the alimentary canal found by Schmidt-Mülheim[1] indicated that excretion lags behind absorption during the first 2 hours, nitrogen being retained in the body during this period. From then till the 12-14th hour absorption and excretion run almost parallel. Absorption is practically finished at this time, but excretion continues at a slow rate, gradually falling to the fasting level.

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[1] Falta: *Deutsch. Arch. f. klin. Med.*, lxxxvi.

[2] Graffenberger: *Zeitschr. f. Biol.*, xxviii, p. 337.

[3] Veraguth: *Journ. of Physiol.*, xxi, p. 112, 1897.

[4] Tschlenoff: *Correspondenz-Blatt f. Schweizer Aerzte*, 1896.

[5] Stauber: *loc. cit.*; *Centralbl. f. d. med. Wiss.*, 1896, p. 349.

[6] Feder: *Zeitschr f. Biol.*, xvii, p. 541.

From the above it appears that under normal conditions the rate of excretion of nitrogen in a dog during the first 12 hours after feeding gives a fairly accurate picture of the course of digestion and absorption. Our own results indicate that, with an animal kept under uniform and normal conditions, the rate of nitrogen excretion is almost entirely dependent upon the food, and characteristic for any given diet. By combining the data concerning the rate of excretion with those from analysis of the feces, we aimed to obtain results showing both the rate and the completeness of the absorption of protein from the different foods employed.

METHODS.

A dog in approximate nitrogenous equilibrium was fed once in 24 hours, and the rate of nitrogen excretion in the urine followed by catheterizing at 3-hour intervals. The rate of excretion is taken as an index of the readiness with which the proteins are digested, absorbed from the alimentary canal, and metabolized in the body. The nitrogen of the feces is taken as an index of the relative completeness with which the proteins of the different foods are absorbed.

For all except the first experiments (Table I) the dog was fed the following diet.

Meat	to contain 3 gm. of nitrogen.
Starch	65 gm.
Fat (Lard + Meat fat)	26-27 gm.
Salt	5 gm.
Bone Ash	5 gm.

During alternate experiments charcoal was added to the food, in order to make possible a separation of the feces from the different diets. With the addition of bone ash this proved satisfactory. The food was warmed to body temperature before feeding.

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[1] Schmidt-Mülheim: *Arch. f. Anat. u. Physiol., Physiol. Abt.*, 1879, p. 39.

The meats were freed from visible fat, skin, etc., and ground as fine as possible in a machine. They were then boiled for 20-25 minutes, and thoroughly drained. The salt cod was shredded and soaked in fresh water over night before boiling. Immediately after taking samples for analysis the meats were frozen, and kept in that condition, as suggested by Gies.

FOOD ANALYSES. Kjeldahl determinations were performed upon 2-gram portions of the ground and drained meats. The samples were taken from different parts of the main portion; duplicates as a rule, agreed satisfactorily. The starch and lard were practically nitrogen free.

For fat determinations, 2 gm. of the meat, without preliminary drying, were ground up with anhydrous copper sulphate until the mixture became a dust-dry powder. This was then extracted 8 hours in a paper thimble with carbon tetrachloride. The method proved convenient and gave close duplicates throughout. The fat contents of the different meats were: periwinkle, 1.78 per cent; squeteague, 9.35 per cent; tautog, 2.03 per cent; eel, 6.79 per cent; boiled cod, 0.50 per cent; fried cod, 1.83 per cent.

ANALYSIS OF FECES. The feces as soon as gathered were placed in concentrated sulphuric acid, in which they formed a solution or homogenous suspension. This was diluted to a known volume at the conclusion of each food test, and aliquot portions used for nitrogen determinations.

URINE was collected chiefly by catheterization, the bladder being washed 4 times at each catheterization. Urine voided in the cage was washed into a bottle containing acid, and united with that obtained by catheterization at the end of the current period. The animal, a fox terrier bitch of 7.3 kilos weight, was catheterized every three hours after the daily feeding for 12 hours, then again at the end of the 24th hour.

RESULTS.

In order to test the effect of non-protein food on the digestibility of protein, the preliminary experiments tabulated in Tables I and II were performed. The animal was not yet in equilibrium. In the first 2 days of the series the dog received daily 60 grams of meat and 25 grams each of crackerdust and lard. During the next two days 60 grams of cornstarch was added to the diet. The effect of the added starch is markedly apparent in a retardation of absorption, as shown by the slower excretion of urinary nitrogen. While with diet 1 the height of excretion occurs in the first two periods, during which 0.47 and 0.69 gram of nitrogen were excreted, with diet 2 only 0.37 and 0.49 gram were voided during these periods, and thereafter the rate remained higher than with diet 1, indicating that a longer time was required for finishing the digestion and absorption when more carbohydrate was present. From these results it is apparent that not only the amount of protein fed, but also that of the other food constituents is a decided factor in the rate of absorption.

The nitrogen of the feces indicated little difference in the completeness of absorption, 84.8 per cent of the nitrogen in one case and 82.3 per cent in the other. The effect of the added starch in sparing protein and bringing about nitrogenous equilibrium is seen on the fourth day.

The remaining experiments were performed after the animal had attained equilibrium on the standard diet (p. 222). The tests were run in duplicate, except for the weakfish and periwinkle. The average results are summarized in Table II, the data from the different diets being arranged in a descending series, based on the relative rate at which the protein in each is digested and absorbed, as measured by the nitrogen excretion in the urine. The nitrogen excreted during the first 9 hours after feeding is taken as the index for comparison. The weight of the dog, remained without significant change during the experiments.

Table II shows a striking and unexpected relation between the relative rates at which the proteins were digested and metabolized, as shown by the nitrogen balances. The diets tabulated at the left, from which the nitrogen was absorbed and metabolized most rapidly, were least capable of maintaining equilibrium. The loss of nitrogen is in three of the four diets showing a negative balance, due at least partly to decreased absorption, as shown by the large nitrogen content of the feces. A possible explanation is that both the rapid digestion and the incomplete absorption were due to stimulation of peristalsis, which caused the alimentary contents to be digested and absorbed more rapidly, as the result of quicker mixing with the digestive fluids and more thorough contact with the absorbing surfaces of the digestive tract, but at the same time passed the contents through too rapidly for complete absorption. Another factor lies in the fact, shown by the recent work of Carrel, Meyer and Levene[1], that protein is more efficient in maintaining nitrogenous equilibrium when it is absorbed before cleavage has

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[1] *Amer. Journ. of Physiol.*, 1909, 1910.

TABLE 1.

Effect of Starch on Rate of Protein Digestion.

8, I, 1910			8, II, 1910				
HOURS	1	PER HOUR	2	PER HOUR		GRAMS	N
0–3	0.4885	0.1614	0.4540	0.1539	Weakfish-flesh	60.00	2.24
3–6	0.6834	0.2278	0.7060	0.2353	Cracker Dust.	25.00	0.44
6–9	0.4150	0.1383	0.4450	0.1483	Lard	25.00	0.00
9–12	0.2941	0.0980	0.2900	0.0967	Salt	5.00	0.00
12–24	0.8360	0.0697	0.8266	0.0689			
Total	2.7170		2.722				
Feces	0.428		0.428				
N excretion	3.145		3.150				
Food N	2.680		2.680				
N Balance	–.465		–.470				

Digestion coefficient, 84.8 per cent.

8, III, 1910			8, IV, 1910				
HOURS	1	PER HOUR	2	PER HOUR		GRAMS	N
0–3	0.3783	0.1261	0.3727	0.1242	Weakfish-flesh	60.00	2.24
3–6	0.5002	0.1667	0.4799	0.1600	Cracker Dust	25.00	0.44
6–9	0.4704	0.1568	0.4423	0.1474	Starch	60.00	0.00
9–12	0.3858	0.1283	0.2809	0.0936	Lard	25.00	0.00
12–24	1.029	0.0857	0.7078	0.0589	Salt	5.00	0.00
Total	2.764		2.283				
Feces	0.473		0.473				
N excretion	3.237		2.756				
Food N	2.680		2.680				
N Balance	–.557		–.076				

Digestion coefficient, 82.3 per cent.

proceeded to the lowest stages. Whatever the cause, it is evident that ready digestibility of protein foods does not indicate complete availability, but that, within certain limits, the relations may be exactly the reverse. The relations between the fresh and salt cod diets form a marked illustration. The nitrogen from the fresh cod, boiled or fried, was digested and absorbed the most rapidly of that of any of the diets, but, absorbed and retained the least completely. The salt cod, which was fed in the interval between the two fresh cod diets, was absorbed and metabolized much more slowly, doubtless due to the physical effect of the preservation in salt, but the absorption and retention were proportionally more complete.

The above results are consistent with those recently obtained by E. Voit and Zisterer,[1] who find that casein is less capable of maintaining nitrogenous equilibrium when fed after artificial digestion with pepsin, than when fed without previous digestion; too early peptonization appeared to decrease the food value of the protein.

TABLE II.

Summary of Mean Results.

FOOD	BOILED COD	FRIED COD	BOILED BEEF	BOILED TAUTOG	BOILED EEL	BOILED WEAK-FISH	BOILED MUSSEL	BOILED SALT COD	BOILED PERI-WINKLE
N in urine during first 9 hours after feeding.	1.50	1.36	1.29	1.28	1.24	1.23	1.23	1.07	1.00
N absorbed in 24 hours......	1.98	1.80	2.58	2.55	1.91	2.53	2.40	2.58	2.57
N excreted in 24 hours	2.51	2.48	2.76	2.35	2.20	2.34	2.22	2.29	1.90
N retained......	−0.53	−0.68	−0.18	+0.20	−0.29	+0.19	+0.18	+0.29	+0.47

SUMMARY.

A dog was once fed in 24 hours and catheterized 3, 6, 9, 12 and 24 hours after each meal. The rate of nitrogen excretion is taken as an index of the rate of absorption from the alimentary canal.

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[1] *Zeitschr. f. Biol.*, liii, p. 457.

Addition of starch to the diet decreased the rate of nitrogen metabolism, but had no significant effect on the completeness of absorption.

The diets containing boiled meats, other constituents being constant, rank as follows when arranged in order according to the relative *rates* at which their nitrogen was digested and absorbed, as indicated by the nitrogen excretion: fresh cod, beef, tautog, eel, weakfish, mussel, salt cod, periwinkle.

When ranked according to the amount of nitrogen *retained* from each, the order is practically reversed.

The failure to retain the nitrogen of the more quickly digested and metabolized proteins appears partly due, in the fresh cod and eel diets at least, to incomplete absorption. Another cause doubtless lies in the fact that a larger proportion of the more rapidly digested proteins is absorbed in the form of the lowest cleavage products, which appear, from recent work of Carrel, Levene, Meyer, and Manson, less capable than the higher cleavage products, of maintaining the nitrogenous equilibrium of the body.

Apparently there is an *optimum rate of digestion* in the alimentary tract, which constitutes the condition for the formation and absorption of proteolytic products in a manner making possible their most complete assimilation by the body. This optimum rate of digestion may not only be fallen short of, but may be exceeded, as in some of the experiments above reported.

III.

Beef

HOURS	8, VII, 1910	8, VIII, 1910
0–3	0.3612	0.3182
3–6	0.5338	0.5038
6–9	0.4470	0.4439
9–12	0.4747	1.363
12–24	0.9870	
0–24	2.804	2.629
N of feces	0.423	0.423
	3.227	3.052
Balance	−0.227	−0.052

IV.

Weakfish (Cynoscion regalis.)

HOURS	8, IX, 1910
0–3	0.3400
3–6	0.5009
6–9	0.3972
9–12	0.3757
12–24	0.8740
0–24	2.488
N of feces	0.472
	2.960
Balance	+0.040

V

Tautog (Tautoga onitis)

HOURS	8, XII, 1910	8, XIII, 1910
0–3	0.3390	0.3150
3–6	0.6110	0.5150
6–9	0.3770	0.4052
9–12	0.3850	0.3161
12–24	0.7618	0.6940
0–24	2.474	2.245
N of feces	0.444	0.444
	2.918	2.689
Balance	+0.082	+0.311

VI

Periwinkle (Litorina litorea.)

HOURS	8, XIV, 1910	8, XV, 1910
0–3	0.2940	0.2880
3–6	0.4660	0.3780
6–9	0.3220	0.2549
9–12	0.2548	0.2507
12–24	0.6534	0.6457
0–24	1.990	1.817
N of feces	0.634	0.634
	2.624	2.451
Balance	+0.376	+0.549

VII

Eel (Anguilla chrysypa.)

HOURS	8, XVI, 1910
0–3	0.3657
3–6	0.5720
6–9	0.3010
9–12	0.3055
12–24	0.6515
0–24	2.196
N of feces	1.087
	3.283
Balance	−.283

VIII

Mussel (Mytilus edulis.)

HOURS	8, XVII, 1910	8, XVIII, 1910
0–3	0.3152	0.2720
3–6	0.4790	0.4480
6–9	0.4930	0.4580
9–12	0.3210	0.3402
12–24	0.6555	0.6570
0–24	2.264	2.175
N of feces	0.604	0.604
	2.868	2.779
Balance	+.132	+.221

IX

riled Cod (Gadus callarias)

OURS	8, XIX, 1910	8, XX, 1910
......	0.2830	0.3580
......	0.6365	0.6560
......	0.5600	0.5095
......	0.3360	0.3105
4.....	0.6370	0.7215
......	2.452	2.555
f feces	1.020	1.020
	3.472	3.575
ance...	−.472	−.575

X

Salt Cod

HOURS	8, XXI, 1910	8, XXII, 1910
0–3.......	0.2645	0.2660
3–6.......	0.4550	0.4558
6–9.......	0.4610	0.3460
9–12......	0.2970	0.3725
12–24	0.7914	0.9430
0–24......	2.269	2.383
N of feces	0.422	0.422
	2.691	2.805
Balance...	+.309	+.195

XI

Fried Cod

HOURS	8, XXIII, 1910	8, XXIV, 1910
0–3.......	0.3623	0.2030
3–6.......	0.6840	0.5015
6–9.......	0.4985	0.4805
9–12......	0.2960	0.4170
12–24.....	0.7080	0.8050
0–24......	2.549	2.407
N of feces	1.197	1.197
	3.746	3.604
Balance...	−.746	−.604

THE INCREASE IN METABOLISM DUE TO THE WORK OF TYPEWRITING.

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INTRODUCTION.

Numerous experiments have been made to determine the metabolism of an individual at rest, either reclining upon a bed or couch, or sitting quietly in a chair. In many of these the carbon dioxide output and oxygen consumption have been measured, in others the heat production and heat elimination, and in a smaller number all four factors simultaneously. From the results of these researches it is possible to compute quite accurately the 24-hour metabolism of an adult individual lying in bed or sitting, combined with a definite number of hours of sleeping. If, however, an attempt is made to compute the total metabolism of an individual engaged in an occupation in which muscular work is involved, or even in common everyday movements such as walking, running, and going up and down stairs, we find that the amount of available data for such a calculation is extremely limited and, indeed, in most instances wholly lacking. While many people in this country, particularly women, are engaged in stenography and typewriting, thereby earning a livelihood, the amount of mechanical work involved in operating a typewriter and the expenditure of energy required have been but little studied. Some idea, however, of the increase in the several factors of metabolism coincident with this type of work was given by the results of two experiments reported in a previous publication.[1] Although the results of these experiments were not wholly satisfactory, the study was not continued owing to the fact that the apparatus used was necessarily dismantled, and no additional experiments were possible for some years. Inasmuch as these were the only experiments which had been made on this subject, it was deemed advisable to publish the results at that time, and to add to the study by making other experiments when opportunity offered. Among the many lines of study upon total metabolism which the Nutrition Laboratory has in view is that of muscular work *per se*, and the amount of muscular work and total metabolism involved in various occupations. As typewriter operators could be easily secured in the vicinity of the Nutrition Laboratory, at the suggestion of the Director, the writer recently carried out the series of experiments herewith reported, in which the increase in total metabolism due to the work of typewriting was studied.

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[1] Carpenter and Benedict: This *Journal*, vi, p. 271, 1909.

APPARATUS.

Respiration calorimeter. The apparatus used in this study for measuring the respiratory exchange and the heat elimination was the respiration calorimeter of the so-called "chair" type, which has been described in detail in a previous publication.[1] This apparatus permits a person to sit comfortably in a chair, and make such movements with the arms and hands as one can perform while in a sitting position. It is admirably adapted for a study like the one here reported. By means of this apparatus it is possible to measure the carbon dioxide eliminated, oxygen absorbed, water vaporized, and heat eliminated in hour periods with a great degree of accuracy. The apparatus has been repeatedly tested by means of alcohol and electrical check experiments and the factors referred to have been determined to within 1 or 2 per cent per hour.

The ventilation of the apparatus in the study here reported was, during the rest periods, about 40 liters per minute; during the typewriting periods this was increased to about 75 liters per minute, thus securing uniformity in composition of the air during the rest and typewriting periods. The humidity in all of the experiments varied from 32 per cent to 55 per cent, while the carbon dioxide varied from 30 parts per 10,000 to 87 parts per 10,000. The temperature was comparatively uniform in the experiments, being kept between 20.5° and 21.5°C.

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[1] Benedict and Capenter: *Carnegie Institution of Washington Publication No. 123*, 1910.

Accessory apparatus. In order to obtain data regarding the physical condition and muscular activity of the subjects, several forms of apparatus were used. The pulse rate was obtained by means of a Bowles stethoscope which was attached to the chest of each subject in such a manner that when connected by means of tubing to the outside of the chamber, a count of the heart beat could be easily obtained. It was possible in this way to get the pulse rate in the rest experiments in a number of instances, although not during the entire experiment. During the typewriting periods, it was extremely difficult to get any records of this factor, the only opportunity for this being when the subjects put a new sheet of paper into the typewriter.. Even at that time, however, the pulse beats which could be heard distinctly were very few, seldom, if ever, more than 10 or 12. By using a stop-watch, an approximate count was obtained of these few pulse beats, and while the individual counts in some of the typewriting periods may be slightly in error, it is believed that the average of a number of separate records is sufficiently accurate. In some of the experiments, however, the stethoscope was pulled away from the chest of the subject in such a manner that it was impossible to get any count. Upon listening at the tube from the stethoscope, it was found that an assistant could note the time at which the carriage was thrown back for the beginning of a new line. Accordingly, in several of the experiments time records were made upon the kymograph record to show the speed at which the work was being done.

An Ellis pneumograph was used in these experiments; first, to obtain a record of the relative muscular activity or quietness of the subject in the rest periods; and second, to get the rate of respiration. The records of the muscular activity were obtained by having the pointer of a tambour write upon a kymograph drum. This kymograph drum, which was 51 cm. in circumference, revolved at a very slow rate, so that one revolution was obtained in about three-quarters of an hour, or in one hour. The minor oscillations of the pointer which could be counted by using a stop-watch showed the respiration, while the major excursions showed the variations in muscular activity, or when the subject took a deep breath.

During the typewriting periods in the earlier experiments, the kymograph was made to revolve at a very much faster rate of about one revolution in 3 to 5 minutes; and by using a Jaquet chronometer, a time line was also obtained, giving seconds simultaneously with the records of the respiration movements. In the later experiments the respiration records were obtained simultaneously with the records of the muscular activity by connecting a glass T to the tube from the pneumograph, one kymograph being revolved at a slow rate, and the other at a somewhat faster rate.

The body temperatures when taken were obtained by means of clinical thermometers inserted underneath the tongue. The thermometer was held in this position with the mouth closed for about 4 minutes; it was then removed, put in a case, and read after the experiment was finished. This record was usually taken at three separate times in an experiment, *i.e.*, during the first 5 minutes of the first rest period, during the first 5 minutes at the beginning of the typewriting period, and after the typewriting period was finished.

Two standard makes of typewriters were used in the experiments, one a recent model of the so-called "visible" type, and the other an older model of the "invisible" type. In order to support the machines a specially constructed wooden table was used which fitted upon the arms of the chair. This permitted the subject a slight freedom of movement and at the same time allowed him to operate the machine without being hampered.

PLAN OF EXPERIMENTS.

The general plan observed in practically all of the experiments in this study was as follows: The subject, who had previously been instructed regarding details and routine, came to the laboratory early on the morning of the experiment, having eaten no breakfast. After the stethoscope and pneumograph had been adjusted, he immediately entered the respiration chamber and seated himself. The typewriter stand, typewriter, and accessory apparatus were then adjusted, the subject commenced reading, and as soon as the calorimeter had been sealed, the preliminary period began. After equilibrium had been established, the experiment was commenced and the several periods of rest followed according to the prearranged program. During these rest periods the subject remained seated, extremely quiet, reading, being particularly quiet at the end of each period in accordance with special signals.[1] Then followed immediately the periods of typewriting. The experiments were subdivided in some cases into three-quarter hour periods, while in others the periods were one hour in length; in one instance no subdivision was made in the typewriting period.

In the typewriting periods, the subjects copied from printed articles, wrote a stereotyped phrase over and over, or wrote material from memory. It was not stipulated that the subjects should write accurately, the idea being to accomplish a considerable amount of the kind of muscular work involved in operating a typewriter. With the female subjects the time of the experiment was arranged so that it did not occur during the period of catamenia and thus the possible influence of this factor was avoided.

SUBJECTS.

The subjects of these experiments were five in number, two men and three women. The statistics regarding sex, age, height, and weight without clothes are given in table 1.

TABLE 1.

Statistical data of subjects.

SUBJECT	SEX	AGE	HEIGHT	WEIGHT WITHOUT CLOTHES
			cm.	*kilos.*
A	Female	22	161	48.1
B	Female	18	166	51.1
C	Male	33	172	72.6
D	Female	31	166	63.6
E	Male	20	176	58.4

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[1] In short periods in experiments of this character it is necessary for the subject to remain absolutely quiet during the last 15 minutes of an experimental period in order to obtain an accurate determination of the oxygen absorption. For a discussion upon the various factors which affect the determination of oxygen with this apparatus see Benedict and Carpenter: *Carnegie Institution of Washington Publication No. 123*, 1910, p. 83.

Two of the subjects, A and B, were members of the staff of the laboratory, and had been subjects in a study of the normal metabolism of women made a little less than a year previous. They had been several times in both the bed and chair calorimeters and were therefore entirely familiar with the routine of a respiration calorimeter experiment. Subject A had had an experience of about four years in operating the typewriter and subject B a year and a half. Subject A wrote on the "sight" principle, while subject B operated her machine upon the "touch" principle. Subjects C, D, and E were entirely unfamiliar with the apparatus or with any experiments performed with it, excepting from previous explanations. Subject C had been out of employment for several months and was somewhat out of practice in typewriting. Subject D had had an experience of seven or more years, while subject E had operated a machine for about one year. Subjects C and D wrote on the "sight" principle while subject E operated the typewriter according to the "touch" method. Care was taken to secure operators of the highest degree of skill and with an ability to write at an unusually rapid rate.

STATISTICS OF EXPERIMENTS.

In the statistical data reported herewith, the experiments are grouped according to subjects. The explanatory data regarding the conduct of the experiments are given in the text, while the results of the measurements of the metabolism and the amount of work done may be found in the tables.

Experiments with Subject A.

EXPERIMENT No. 1, DECEMBER 13, 1910.

The subject arrived at the laboratoy on the morning of the experiment at 7.25 a.m. and entered the chamber at 7.35 a.m. The preliminary period ended at 8.18 a.m. and was followed by two rest periods of three-quarters of an hour each. At 9.50 a.m. the typewriting was begun and continued steadily without interruption for two hours, at the end of which time the experiment was finished. She stated that while the air seemed rather close and she felt quite warm, especially during the period of work, the experience was not disagreeable. In this experiment the subject wore a heavy sweater besides her usual clothing; furthermore, the rate of ventilation was not increased during the typewriting as was done in later experiments. These facts may have accounted for the unusual warmth. Although somewhat nervous at the beginning of the typewriting period, the subject said this soon wore off. The work did not seem hard, and she said she could have written another hour without becoming tired. A part of the time the subject copied from scientific articles and the rest of the period wrote phrases from memory or of her own composition.

The results of the measurement of the total metabolism are given in table 2 and the record of the amount of typewriting in table 3.

TABLE 2.

Measurements of metabolism by periods in experiments with subject A.

DATE AND PERIOD	CARBON DIOXIDE ELIMINATED		OXYGEN ABSORBED		RESPIRATORY QUOTIENT	WATER VAPORIZED	HEAT ELIMINATED	AVERAGE PULSE RATE	AVERAGE RESPIRATION RATE
	Total	Per minute	Total	Per minute					
	grams	*cc.*	*grams*	*cc.*		*grams*	*cals.*		
December 13, 1910									
Resting									
8.18–9.03 a.m.	14.5	164	11.7	182	0.90	10.7	38.9	79	19
9.03–9.48 a.m.	15.2	172	14.3	223	0.77	11.5	42.6	85	–
Typewriting									
9.48–11.48 a.m.	59.3	252	52.0	303	0.83	44.8	158.2	121	–
December 28, 1910									
Resting									
8.31– 9.16 a.m.	13.6	153	11.5	179	0.86	12.6	43.5	78	20
9.16–10.01 a.m.	14.2	160	12.6	196	0.82	13.5	45.7	76	19
Typewriting									
10.01–10.46 a.m.	22.0	248	20.2	314	0.79	16.9	50.0	–	30
10.46–11.31 a.m.	21.0	238	20.3	316	0.75	18.9	60.2	–	29
11.31 a.m.–12.16 p.m.	21.5	242	19.8	308	0.79	19.3	60.0	–	29*

*Records of respiration after experiment: 19; 18; 19; 21; 22.

TABLE 3.

Amount of work performed in experiments with subject A.

DATE	NUMBER OF PERIOD	LENGTH OF PERIOD*	NUMBER OF WORDS	NUMBER OF STROKES	NUMBER OF LINES
1910					
December 13.	1	2 hrs.	7634	39673	477
Dec. 28.	1	45 min.	2773	12960	168
	2	45 min.	3476	15751	210
	3	45 min.	3673	16135	212

*The period of actual typewriting was somewhat less. See text.

As the pneumograph was not firmly adjusted, it was possible to obtain only two records of the respiration, both of these during the first rest period. The average pulse rates are the result of four observations in the first rest period and six in the second, the range for the two periods being from 76 to 90. During the work period it was not possible to hear the heart-beat for more than 6 to 14 beats at a time, but by taking the time with a stop-watch, the rate per minute could be calculated. There were eight counts thus taken and while the individual counts, which ranged from 100 to 135 per minute, may not be exact, it is believed that the average of 121 represents the pulse rate per minute during typewriting. One observation immediately after the subject had stopped typewriting gave a result of 141 per minute.

EXPERIMENT No. 2, DECEMBER 28, 1910.

The subject arrived at the laboratory at 7.15 a.m. and at 7.35 a.m. entered the respiration calorimeter. The experiment began at 8.31 a.m., the two first periods of three-quarters of an hour each being rest periods. The typewriting period was supposed to begin at 10.01 a.m. but owing to a misunderstanding, the subject did not begin typewriting until 10.12 a.m. The subject then continued writing steadily until 12.16 p.m., the time being divided into three three-quarter hour periods as in the rest experiment. At the end of each period she stopped long enough to lay aside the typewritten sheets and mark them for the different periods. At the end of the typewriting period, the subject left the calorimeter.

In this experiment the subject did no copying but wrote material of her own composition or a set phrase, repeated over and over. She stated that it was difficult to keep awake during the rest period and extremely tiring during the work period. This was due, she thought, to the fact that during several nights preceding the experiment she had not had her accustomed amount of sleep and for that reason was very tired when she began the experiment in the morning. In her typewritten material she mentioned the fact that her wrists became very tired and that she relieved the tired feeling by changing the phrases she was using and giving certain fingers a rest for a short time. She said that the air in the chamber did not seem so close as in the previous experiment. Her clothing was very warm when she came out of the chamber and she had the appearance, also, of having perspired somewhat during the experiment.

The results of the measurements of metabolism by periods are given in table 2, and the amount of typewriting performed in table 3.

During the first rest period, 11 observations of the pulse were taken, and during the second rest period, 8 observations. The range in the two rest periods was from 72 to 83 per minute. It was impossible to get the pulse rate in the working period because of the noise of the machine and the fact that the subject wrote somewhat faster than in the previous experiment. However, three observations were made immediately after she ceased typewriting and these were found to be 100, 86, and 90 per minute respectively.

During the rest period a number of counts of the respiration rate were made, averaging 20 and 19 in the two periods. During the typewriting period a very large number of observations were obtained graphically, simultaneously with a time line; these were subsequently counted and the averages are given in the table.

The sublingual body temperatures obtained at the beginning and end of the resting periods and at the end of the typewriting periods in experiment 2 were 97.9°F., 98.1°F. and 98.7°F., respectively; the heat production calculated from these changes in body temperature was 93.8 calories for the rest periods and 184.0 calories for the typewriting periods.

An explanation of table 3, which will apply to all similar tables, should be given here. The column "Number of words" gives the number of words written during the periods designated, each word being counted regardless of whether it was correctly spelled or not. The data in the column "Number of strokes" were obtained by counting both the letters, and the spaces between words in each line; the figures thus represent the number of times the keys or spacing bar were struck. The number of lines shows the number of times the carriage was thrown back in order to begin a new line.

Experiments with Subject B.

EXPERIMENT No. 1, DECEMBER 15, 1910.

The subject arrived at the laboratory at 7.10 a.m., and at 7.25 a.m. entered the respiration calorimeter. The experiment began at 8.29 a.m., the first two three-quarter hour periods being rest periods. At 9.59 a.m., the typewriting period began and continued for two hours. During this time the subject wrote steadily except for stopping long enough at the end of the first hour to lay aside the sheets on which she had written. The experiment ended at 11.59 a.m.

The subject copied from scientific articles for the most part but also wrote poetry from memory. She said that the time passed very quickly, especially during the rest period. At the beginning of the first period of typewriting she became extremely warm and this continued through practically the whole period; during the second period of typewriting she was not so warm, but was simply comfortable. She did not find the experiment particularly fatiguing.

The results of the metabolism as measured by periods are given in table 4, and the amount of work done during the typewriting periods in table 5.

During the first rest period there were 5 observations of the pulse rate taken, and 7 observations during the second rest period, with a range for the whole rest period of 93 to 100. During the typewriting period the pulse could be heard quite distinctly. In the first hour, 8 observations were obtained with a range of 103 to 113, and in the second hour 6 observations, with a range of 108 to 117. Immediately after the experiment was over and the subject had finished typewriting, one count was obtained, giving a result of 95 per minute.

TABLE 4.

Measurements of metabolism by periods in experiments with subject B.

DATE AND PERIOD	CARBON DIOXIDE ELIMINATED		OXYGEN ABSORBED		RESPIRATORY QUOTIENT	WATER VAPORIZED	HEAT ELIMINATED	AVERAGE PULSE RATE	AVERAGE RESPIRATION RATE
	Total	Per minute	Total	Per minute					
	grams	*cc.*	*grams*	*cc.*		*grams*	*cals.*		
December 15, 1910.									
Resting									
8.29–9.14 a.m.	15.6	177	12.7	198	0.89	11.5	43.4	96	17
9.14–9.59 a.m.	16.1	182	14.5	226	0.81	11.8	43.4	97	17
Typewriting									
9.59–10.59 a.m.	29.2	248	26.0	304	0.82	22.3	73.3	108	22
10.59–11.59 a.m.	28.7	244	27.4	319	0.76	23.7	79.2	112	-
December 21, 1910.									
Resting									
8.45– 9.30 a.m.	15.2	172	14.5	226	0.76	12.8	44.6	88	15
9.30–10.15 a.m.	14.7	167	13.7	212	0.79	13.8	43.5	89	15
Typewriting									
10.15–11.15 a.m.	28.3	240	26.1	305	0.79	24.2	70.9	109	-
11.15 a.m.–12.15 p.m.	26.8	227	24.8	289	0.79	25.9	74.9	115	21

TABLE 5.

Amount of typewriting performed in experiments with subject B.

DATE	NUMBER OF PERIOD	LENGTH OF PERIOD*	NUMBER OF WORDS	NUMBER OF STROKES	NUMBER OF LINES
		hour			
December 15	1	1	3413	19604	248
	2	1	3275	19129	250
December 21	1	1	3641	19415	248
	2	1	3475	18418	234

* The period of actual typewriting was somewhat less. See text.

A number of observations of the respiration rate were also secured—4 in the first rest period, 5 in the second, and 6 in the first typewriting period.

Experiment No. 2, December 21, 1910.

The subject came to the laboratory at 7.40 a.m., and at 7.48 a.m. entered the respiration chamber. The experiment began at 8.45 a.m. and two rest periods of three-quarters of an hour each followed. At 10.15 a.m. the first typewriting period commenced, the subject beginning to write at 10.16 a.m. She continued typewriting steadily for two hours, excepting at the end of the first hour when she laid aside the sheets previously used. The experiment was finished at 12.15 p.m. During the whole typewriting period, the subject copied from a scientific article. Her observations regarding the experiment itself were not unlike those regarding the previous experiment; she was not particularly tired and in no way suffered from the experience.

The results of the measurements of metabolism by periods are given in table 4, and the amount of work done in the two hours of typewriting in table 5.

During the first rest period 8 observations of the pulse rate were obtained, and during the second, 6 observations, with a total range of 82 to 94. During the first typewriting period 4 observations of the pulse rate were obtained and during the second, 7 observations, with a total range of 102 to 122. During the first rest period, 7 observations of the respiration were made, 5 in the second, and 7 during the period of typewriting.

In experiment No. 1, the sublingual body temperatures at the beginning and end of the rest periods and at the end of the typewriting periods were 98.8° F., 98.9° F., and 98.9° F., respectively, and at corresponding times in experiment No. 2, 98.35° F., 98.35° F., and 98.4° F. The heat production calculated upon these changes in body temperature was as follows; Experiment No. 1, resting period, 89.1 calories, typewriting period, 152.5 calories; experiment No. 2, resting period, 88.1 calories, typewriting period, 147.1 calories.

Experiments with Subject C.

EXPERIMENT No. 1, DECEMBER 27, 1910.

The subject arrived at the laboratory at 8.08 a.m., and entered the respiration chamber at 8.30 a.m. The experiment began at 9.19 a.m., followed by three rest periods of three-quarters of an hour each. At 11.34 a.m., the typewriting period began. The subject commenced typewriting at 11.38 a.m. and continued steadily for 3 hours, except when he used the telephone at 12.52 p.m., at which time the light inside of the respiration chamber was out. The experiment was finished at 2.34 p.m. During the entire time of typewriting, the subject wrote one phrase of 16 words, shifting the carriage each time the phrase was completed. The subject stated that he was notuncomfortable during the experiment and that it was neither too cold nor too warm inside of the chamber; that he was not hungry; and although a little annoyed by the light going out several times he got along very well.

The results of the measurements of metabolism by periods are given in table 6 and the amount of work performed in table 7.

During the first rest period, 10 observations of the pulse rate were obtained with a range of 84 to 90, and an average of 87. During the remainder of the experiment it was impossible to obtain the pulse rate as the stethoscope had become displaced.

A large number of observations of the respiration rate were obtained, and these will be discussed later.

TABLE 6.

Measurements of metabolism by periods in experiments with subject C.

DATE AND PERIOD	CARBON DIOXIDE ELIMINATED		OXYGEN ABSORBED		RESPIRATORY QUOTIENT	WATER VAPORIZED	HEAT ELIMINATED	AVERAGE PULSE RATE	AVERAGE RESPIRATION RATE
	Total	Per minute	Total	Per minute					
	grams	*cc.*	*grams*	*cc.*		*grams*	*cals.*		
December 27, 1910.									
Resting									
9.19–10.04 a.m.	20.3	230	18.0	280	0.82	13.2	54.4	87	17
10.04–10.49 a.m.	19.2	217	15.0	233	0.93	13.9	51.1	-	16
10.49–11.34 a.m.	19.7	223	18.3	284	0.79	14.4	54.9	-	17
Typewriting									
11.34 a.m.–12.34 p.m.	42.2	358	35.3	412	0.87	31.4	94.5	-	-
12.34 p.m.–1.34 p.m.	44.1	375	38.3	447	0.84	40.6	120.4	-	[illegible]
1.34 p.m.–2.34 p.m.	45.4	385	39.9	466	0.83	43.3	128.5	-	[illegible]
December 29, 1910.									
Resting									
8.40– 9.25 a.m.	19.2	217	15.9	248	0.87	14.1	58.6	76	16
9.25–10.10 a.m.	19.1	216	17.8	278	0.78	14.1	57.0	81	17
10.10–10.55 a.m.	19.6	222	17.4	270	0.82	13.9	54.0	-	-
Typewriting									
10.55–11.55 a.m.	40.2	341	33.1	386	0.88	28.7	86.3	-	25
11.55 a.m.–12.55 p.m.	38.9	330	29.5	344	0.96	33.9	105.3	-	24
12.55 p.m.– 1.55 p.m.	40.4	343	41.0	478	0.72	35.9	115.9	99	[illegible]

TABLE 7.

Amount of typewriting performed in experiments with subject C.

DATE	NUMBER OF PERIOD	LENGTH OF PERIOD*	NUMBER OF WORDS	NUMBER OF STROKES	NUMBER OF LINES
1910		*hours*			
December 27	-	3	12129	49264	758
December 29	1	1	4283	17120	268
	2	1	4281	17088	267
	3	1	4267	17088	267

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* The period of actual typewriting was somewhat less. See text.

EXPERIMENT No. 2, DECEMBER 29, 1910.

The subject came to the laboratory at 7.10 a.m., and entered the respiration chamber at 7.26 a.m. The experiment began at 8.40 a.m., the three first periods of three-quarters of an hour each being rest periods. These rest periods were followed by three typewriting periods, each one hour long, the first beginning at 10.55 a.m. The subject wrote for the 3 hours continuously without interruption, except that at the end of each hour he laid aside the typewritten sheets for that period.

The subject stated that he would not care to repeat the experiment as he found it too hard work to write at such speed for so long a time. During the last hour of typewriting he was extremely tired and said that if he stopped even for a few seconds, it was very difficult to keep the typewriting in mind. His hand and wrist, particularly one arm, became very tired; in fact, it was very difficult to keep up his speed during the last hour. He was more tired than in his previous experiment, did not seem to stand it so well, and was quite hungry. As to the rate of typewriting, he believed that it was uniform throughout the three hours. There was ample room in the chamber for typewriting and in no way was he hampered in his work. As in the preceding experiment, he wrote the same phrase over and over.

The results of the measurement of the metabolism by periods are given in table 6 and the amount of work accomplished during the periods of typewriting is given in table 7.

In this experiment it was not possible to get many observations of the pulse, even during the rest periods. Only 3 observations were made during the first period, with a range of 72 to 79, and during the second period only one observation. In the typewriting period, one observation was taken of 99 per minute.

Five observations of the respiration rate were made during the first rest period and 3 during the second, while a large number were obtained during the typewriting period by means of the records on the kymograph.

The sublingual body temperature for the two experiments was as follows: Experiment No. 1, beginning of rest period, 98.3° F., end of rest period, 98.2°F., end of typewriting period, 99.0° F.; experiment No. 2 at corresponding times, 97.8° F., 97.6° F., and 98.35° F.

The heat production calculated upon these changes in body temperature was as follows: Experiment No. 1, rest period, 157 calories; typewriting period, 372.9 calories; experiment No. 2, rest period, 162.3 calories; typewriting period, 335.5 calories.

Experiment with Subject D.

EXPERIMENT No. 1, JANUARY 8, 1911.

The subject came to the laboratory at 8.15 a.m., and entered the respiration calorimeter at 8.22 a.m. The experiment began at 9.01 a.m. and continued for three three-quarter hour rest periods; the first typewriting period commenced at 11.16 a.m. The subject actually began type writing at 11.21 a.m.

and continued for three three-quarter hour periods, without interruption, except that at the end of each period she laid aside the sheets used during the period. The experiment ended at 1.31 p.m.

The subject stated that she did not find the experiment particularly hard nor was she uncomfortable in any way. She was annoyed somewhat during the typewriting by the fact that a bottle of water on a shelf near her kept jarring off; she finally put it on the chair, where the stopper became loosened and caused some trouble. Aside from this, however, the experiment so far as she was concerned went very well, and was not particularly fatiguing. The air in the respiration chamber was not too cold; in fact, she felt somewhat warm, particularly during the last hour. The typewriting consisted of the phrase used by the previous subject, which was written over and over during the entire period.

The results of the measurements of metabolism are given in table 8, and the amount of work accomplished in table 9.

TABLE 8.

Measurements of metabolism by periods in the experiments with subject D.

DATE AND PERIOD	CARBON DIOXIDE ELIMINATED		OXYGEN ABSORBED		RESPIRATORY QUOTIENT	WATER VAPORIZED	HEAT ELIMINATED	AVERAGE PULSE RATE	AVERAGE RESPIRATION RATE
	Total	Per minute	Total	Per minute					
	grams	*cc.*	*grams*	*cc.*		*grams*	*cals.*		
January 8, 1911.									
Resting									
9.01- 9.46 a.m.	16.9	191	16.5	257	.74	13.8	51.6	96	17
9.46-10.31 a.m.	16.0	181	16.5	257	.71	14.3	50.6	96	19
10.31-11.16 a.m.	15.9	179	15.2	237	.76	15.9	48.7	92	19
Total, 2¼ hours	48.8	184	48.2	250	.74	44.0	150.9	-	-
Typewriting									
11.16 a.m.-12.01 p.m.	26.4	298	-	-	-	23.1	73.3	-	30
12.01 p.m.-12.46 p.m.	27.5	311	-	-	-	26.1	79.8	109	29
12.46 p.m.-1.31 p.m.	25.4	287	-	-	-	26.5	78.2	115	29
Total, 2¼ hours	79.3	299	-	-	-	75.7	231.3	-	-

TABLE 9.

Amount of typewriting performed in experiment with subject D.

DATE	NUMBER OF PERIOD	LENGTH OF PERIOD*	NUMBER OF WORDS	NUMBER OF STROKES	NUMBER OF LINES
		min.			
January 8, 1911	1	45	4565	18256	235
	2	45	5047	20298	279
	3	45	5075	20338	304

* The period of actual typewriting was somewhat less. See text.

In the first period of rest, 11 observations of the pulse were obtained and 9 observations each during the second and third periods. The range during the entire rest period was from 87 to 100. Two counts were obtained during the typewriting period, one at 12.03 p.m., 109; and at 12.47 p.m., 115. It was not possible to obtain others.

A large number of observations of the respiration rate were obtained during the typewriting period, as well as during the rest period.

The body temperatures at the beginning and end of the rest period and at the end of the typewriting period were 98.8° F., 98.7° F., and 98.9°F.; the total heat production for the rest periods and for the typewriting periods was 148.1 and 237.4 calories, respectively.

Experiment with Subject E.

EXPERIMENT No. 1, JANUARY 15, 1911.

The subject came to the laboratory at 7.28 a.m., and entered the respiration chamber at 7.50 a.m. The experiment began at 8.37 a.m., the first three periods of three-quarters of an hour each being rest periods. The typewriting part of the experiment began at 10.52 a.m., being divided into three three-quarter hour periods. The subject stopped at the end of each period to separate the sheets upon which he had written.

This subject, like the two preceding ones, wrote the same phrase over and over during most of the time, and in the rest of the time wrote a short letter. He stated that he got along very well throughout the entire time, although during the last 10 minutes he found his hands were somewhat cramped and it was very difficult to continue writing. It seemed to him that he wrote more during the second period than in the preceding period. In the last half hour he said he felt very warm but was not otherwise uncomfortable and there appeared to be plenty of room inside of the chamber for writing.

The results of the measurements of metabolism by periods are given in table 10 and the amount of typewriting in table 11.

It was possible to obtain with this subject only 8 observations of the pulse rate, all during the first period. These averaged 67, and ranged from 65 to 67. A large number of observations of the respiration rate were obtained.

The records of the body temperatures taken at the usual times were 98.3° F., 97.9° F., and 98.0° F. The total heat production during the rest and typewriting periods were 156.4 and 211.7 calories, respectively.

Summary of work of typewriting in experiments with five subjects.

For purposes of comparison the amount of work which was done by the five subjects in the different experiments has been summarized in table 12, which gives the number of words and strokes per

TABLE 10.

Measurements of metabolism by periods in experiment with subject E.

DATE AND PERIOD	CARBON DIOXIDE ELIMINATED		OXYGEN ABSORBED		RESPIRATORY QUOTIENT	WATER VAPORIZED	HEAT ELIMINATED	AVERAGE PULSE RATE	AVERAGE RESPIRATION RATE
	Total	Per minute.	Total	Per minute.					
January 15, 1911.	*grams*	*cc.*	*grams*	*cc.*		*grams*	*cals.*		
Resting									
8.37- 9.22 a.m......	18.8	212	14.8	230	0.92	15.3	52.3	67	18
9.22-10.07 a.m......	18.9	213	16.8	261	0.82	15.6	61.0	—	18
10.07-10.52 a.m......	19.0	214	15.2	236	0.91	16.3	54.8	—	18
Total, 2¼ hours.......	56.7	214	46.8	243	0.88	47.2	168.1	—	—
Typewriting									
10.52-11.37 a.m......	24.8	280	22.7	353	0.79	22.2	64.0	—	22
11.37 a.m.-12.22 p.m.	24.5	277	21.1	328	0.84	25.5	71.7	—	22
12.22 p.m.-1.07 p.m...	23.0	260	22.6	351	0.74	26.1	73.3	—	21
Total, 2¼ hours.......	72.3	273	66.4	344	0.79	73.8	209.0	—	—

TABLE 11.

Amount of typewriting performed in experiment with subject E.

DATE	NUMBER OF PERIOD	LENGTH OF PERIOD*	NUMBER OF WORDS	NUMBER OF STROKES	NUMBER OF LINES
		min.			
January 15, 1911...........	1	45	3677	15007	237
	2	45	4116	16738	264
	3	45	4125	16796	269

*The period of actual typewriting was somewhat less. See text.

minute. The number of strokes per word ranged from 4 to 6, and averaged 4.5, the words being for the most part short. Inasmuch as the test was based not upon the popular standard of the number of words, but upon the number of strokes, it does not seem advisable to include the data regarding the number of strokes per word in this table. In computing the data in the column "Strokes per minute," the decimals were discarded and the nearest whole number taken, so that if the average number of words per minute as given in this table were multiplied by the number of strokes per minute, the result would be a little larger than that given in the individual tables.

TABLE 12.

Summary of the work of typewriting in experiments with five subjects.

SUBJECT	DATE	PERIOD	WORDS PER MINUTE	STROKES PER MINUTE*
	1910			
A........	December 13...	Total, 2 hrs.....	65	336
	December 28...	1st...............	82	381
		2nd..............	77	350
		3rd..............	82	359
		Total, 2¼ hrs.....	80	362
B........	December 15...	1st...............	59	338
		2nd..............	55	319
		Total, 2 hrs......	57	328
	December 21...	1st...............	62	336
		2nd..............	58	310
		Total, 2 hrs......	60	323
C........	December 27...	Total, 3 hrs......	68	277
	December 29...	1st...............	78	311
		2nd..............	71	285
		3rd..............	71	285
		Total, 3 hrs......	73	293
	1911			
D........	January 8......	1st...............	117	466
		2nd..............	112	451
		3rd..............	116	464
		Total, 2¼ hrs.....	115	460
E........	January 15.....	1st...............	94	385
		2nd..............	91	372
		3rd..............	92	373
		Total, 2¼ hrs.....	92	376

*The average number of strokes per word for each period ranged from 4 to 6; the average number for all the experiments was 4.5.

DISCUSSION OF RESULTS.

In discussing the results obtained in this series of experiments, the effect of the work of typewriting upon the more obvious physiological indexes, *i.e.*, respiration rate, pulse rate, and body temperature, are first considered, then the increase in total metabolism in the different experiments is compared on several bases of comparison. The equivalent amount of work in kilogrammeters is discussed; a comparison is also made with the earlier typewriting experiments.

Respiration rate.—In all of the experiments it was possible to obtain records of the respiration rate per minute, at least in the rest periods, and in many experiments, records were also obtained during the period of typewriting. The rates of respiration during the rest periods were on the whole quite normal, varying from 15 to 20 per minute. With subject A, records were obtained in but one of the typewriting periods. These individual records show that almost as soon as the subject began typewriting, the respiration rate increased from about 17 per minute during rest to 27 per minute during typewriting and this very soon increased to 30 per minute. During the second typewriting period, it fell to 27, then rose again, going as high as 35 per minute in one instance. Throughout the whole typewriting period, an average of 29 per minute was maintained. With the subject B, in the first experiment but very few records were obtained during the typewriting period. The average rate during rest was 17 per minute, while during typewriting it rose to 22 per minute. In the second experiment, the average was 15 per minute during the rest period and 21 during the typewriting. With subject C, the difference between the rest and typewriting periods was greater than with subject B. In the first experiment the average during the rest period was about 17 per minute and during typewriting this rose to 28 and 30 per minute. In one instance it was as high as 38 per minute, and several times rose as high as 35. In the second experiment the average during the rest periods was also 17 per minute; during the typewriting, the averages were 25, 24, and 26 for the three successive periods, the highest record being 30 per minute. For subject D, the averages during the rest periods were 17, 19 and 19, and during the typewriting periods, 30, 29, and 29 respectively. The respiration rate increased to the highest level, about 30, almost as soon as work commenced and remained fairly uniform through the whole time of typewriting. A few records were obtained as high as 35 per minute. With subject E the respiration rate averaged 18 throughout the rest period, while in the typewriting periods the averages increased to 22, 22, and 21. It is thus seen that in practically all of the experiments there was a decided increase in the respiration rate during the typewriting period, showing that so far as may be indicated by the frequency of the respiration, enough muscular work was involved to result in an increased ventilation.

Pulse rate.—But few satisfactory records of the pulse rate were obtained in any of the experiments; in some instances, indeed, the pulse rate may be considered abnormal for a subject sitting quietly at complete rest. Subjects A, B, and C all had rather high pulse rates in the resting periods, particularly subjects B and C. Subject E on the contrary showed a very low pulse rate which was unquestionably no higher than normal for this individual. The high pulse rates of the other subjects may have been due to anxiety regarding the results of the experiments, as in several instances, the subjects stated that a doubt of their ability to write for so long a time at such a high speed made them nervous regarding the success of the experiment. The high pulse rate was probably not due to apprehensiveness regarding their stay in the chamber; this was certainly true of subjects A, and B, as they had been subjects of previous experiments and were perfectly familiar with the apparatus.

During the typewriting periods it was difficult to obtain the pulse records and the observations were accordingly more limited in number than during the rest periods. With subject A several observations were made in the first experiment, giving an average of 121 per minute; one count obtained after the experiment was over showed a pulse rate as high as 141. After the second experiment, when the subject had finished typewriting, successive counts were made showing a pulse rate of 100, 86, and 90, respectively, indicating that the pulse fell very rapidly as soon as the work was finished. With subject B, in the typewriting periods of the first experiment the pulse rose to 108 and 112, and in the second to 109 and 115. One record after the first experiment gave a result of 95 per minute, again showing the fact that the pulse falls very rapidly after the work ceases. With subject D only two records were obtained during the typewriting periods, one of 109 and the other 115, while with subject E, no pulse records were obtained during the work period. From these records it is seen that the work in these experiments was sufficient to raise the pulse rate very noticeably, the increase ranging from about 15 to 40 counts per minute.

Body temperature.—In these experiments the body temperature was obtained with a clinical thermometer sublingually only at the beginning and end of the rest periods and at the end of the typewriting period. With only one subject was there a marked rise. These observations are not considered of very much value, but simply indicate in a general way what the changes in temperature may be under the conditions of the experiments.

The elimination of nitrogen in the urine.—In all the experiments the period for the collection of the urine began an hour or more before the subject entered the chamber and ended at the close of the experiment, but no attempt was made to collect the urine in shorter periods for the purpose of studying the effect of muscular work upon nitrogen elimination. The nitrogen was determined in all the samples and the results are given in table 13, together with the volume and the amount of nitrogen per hour.

TABLE 13.

The elimination of nitrogen in the urine.

SUBJECT	DATE	PERIOD	AMOUNT OF URINE	NITROGEN	
				Total	Per Hour
	1910		*cc.*	*grams*	*grams*
A.....	December 13	7.30 a.m.-12.00 a.m.	86	1.28	0.284
	December 28	7.30 a.m.-12.25 p.m.	72	1.14	0.232
B.....	December 15	7.15 a.m.-12.20.p.m.	66	0.99	0.195
	December 21	7.00 a.m.-12.30 p.m.	62	0.98	0.178
C.......	December 27	7.30 a.m.- 2.45 p.m.	447	3.18	0.439
	1911				
D.......	January 8	8.20 a.m.-1.40 p.m.	153	1.87	0.351
E.......	January 15	6.30 a.m.-1.20 p.m.	230	3.48	0.509

Increase in metabolism per hour due to typewriting.—Since there is considerable muscular exercise involved in operating a typewriter the work of typewriting naturally results in an increase in the total metabolism. The amount of this increase, as well as the percentage above the metabolism during rest, has been calculated and the results are presented in table 14. The hourly metabolism in rest has also been computed for each individual, together with the hourly metabolism during the work of typewriting for each individual experiment; the difference between these two results is divided by the metabolism during rest, thus giving the percentage increase over rest due to typewriting in the individual experiments.

TABLE 14.

Metabolism per hour and increase during typewriting compared with rest.

SUBJECT	DATE		CARBON DIOXIDE ELIMINATED	OXYGEN ABSORBED	WATER VAPORISED	HEAT ELIMINATED	HEAT PRODUCED
	1910		*grams*	*grams*	*grams*	*cals.*	*cals.*
A....	December 13...	Rest..........	19.8	17.3	14.8	54.3	—
		Typewriting..	29.7	26.0	22.4	79.1	—
		Increase......	9.9	8.7	7.6	24.8	—
		Percentage increase.....	50	50	51	46	—
	December 28...	Rest..........	18.5	16.1	17.4	59.5	62.5
		Typewriting..	29.6	27.8	25.1	77.1	83.5
		Increase......	11.1	11.7	7.7	17.6	21.0
		Percentage increase.....	60	73	44	30	34
B....	December 15...	Rest..........	21.1	18.1	15.5	57.9	59.3
		Typewriting..	29.0	26.7	23.0	76.3	76.3
		Increase......	7.9	8.6	7.5	18.4	17.0
		Percentage increase.....	37	48	48	32	29
	December 21...	Rest..........	19.9	18.8	17.7	58.7	58.7
		Typewriting..	27.6	25.5	25.1	72.9	73.6
		Increase......	7.7	6.7	7.4	14.2	14.9
		Percentage increase....	39	36	42	24	25

Considering the experiments individually, it will be seen that in some cases the increase in all four factors was fairly uniform, as for example, in the experiment with subject A on December 13, in which the increase was remarkably uniform, and in the experiments with subject C, in which the percentage increase in the heat production agrees fairly well with the other factors. In some of the other experiments, the agreement was not so marked, however, and it will be noted that the increase in the percentage of the carbon dioxide eliminated during typewriting over that eliminated

TABLE 14—CONTINUED

SUBJECT	DATE		CARBON DIOXIDE ELIMINATED	OXYGEN ABSORBED	WATER VAPORIZED	HEAT ELIMINATED	HEAT PRODUCED
	1911		*grams*	*grams*	*grams*	*cals.*	*cals.*
C....	December 27...	Rest..........	26.3	22.8	18.4	71.3	69.8
		Typewriting..	43.9	37.8	38.4	114.5	124.3
		Increase......	17.6	15.0	20.0	43.2	54.5
		Percentage increase.....	67	66	109	61	78
	December 29...	Rest..........	25.7	22.7	18.7	75.4	72.1
		Typewriting..	39.8	34.5	32.8	102.5	111.8
		Increase......	14.1	11.8	14.1	27.1	39.7
		Percentage increase.....	55	52	75	36	55
D....	January 8......	Rest..........	21.7	21.4	19.6	67.1	65.8
		Typewriting..	35.2	—	33.6	102.8	105.5
		Increase......	13.5	—	14.0	35.7	39.7
		Percentage. increase.....	62	—	71	53	60
E....	January 15.....	Rest..........	25.2	20.8	21.0	74.7	69.5
		Typewriting..	32.1	29.5	32.8	92.9	94.1
		Increase......	6.9	8.7	11.8	18.2	24.6
		Percentage increase.....	27	42	56	24	35

during rest varied from 27 to 67 per cent, while the oxygen absorbed varied from 36 per cent to 73 per cent. The largest variation will be noted in the heat production, which ranged from 25 per cent to 78 per cent. This large variation in the heat production is in all probability due to the fact that the entire amount of heat produced was not actually measured, for it is reasonable to suppose that the increase in body temperature in most of the experiments was greater than was indicated by the clinical thermometer reading. The results for the heat production as given are accordingly in most cases too small, with the possible exception of those in the experiments with subject C. In the experiments with this subject, since the percentage increase in heat production agrees quite well with the percentage increase in the other factors, it would appear as though practically all the heat production in this experiment was actually measured.[1]

For an accurate measurement of the heat production, an accurate measurement of body temperature is absolutely necessary. For this reason in most experiments in this laboratory it is the custom to measure the body temperature very accurately by means of a rectal thermometer, which gives temperatures to within 0.01° C. This was wholly impracticable in these experiments, however, and therefore the heat production is only approximately measured in most instances. Consequently, the increases in the heat production, the carbon dioxide elimination, and the oxygen absorption are not strictly comparable in most of the experiments.

The increase in the water vaporized varies with the other factors excepting in the first experiment with subject C. In this experiment, the increase was very markedly different from the increase in the other factors. It should be stated, also, that in this experiment there was a condensation upon the heat-absorbing apparatus inside the chamber, so that even the increase of 109 per cent does not represent the total increase in the water vaporized.

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[1] The fact should not be overlooked that the accuracy of indirect calorimetry in short experimental periods is yet to be demonstrated; while the calorific equivalents of carbon dioxide and oxygen have both been determined with considerable accuracy for long periods, any comparisons made for periods as short as these must of necessity be of a tentative nature. See discussion of this point in Benedict and Carpenter: *Carnegie Institution of Washington Publication No. 126*, p. 218; and Benedict and Joslin: *Carnegie Institution of Washington Publication No. 136*, p. 188.

These variations in the percentage increases of the factors of metabolism when compared with one another may be due to at least three causes. First, the normal metabolism of the individuals may vary with the different individuals; consequently, if the metabolism was different, assuming that the expenditure of energy involved in writing 1000 words was the same with all individuals, the percentage increase would be different owing to the fact that the base line was different with each individual. For instance, it will be seen that the carbon dioxide elimination for the different subjects during rest varies from 18.5 grams per hour to 26.3 grams. Similarly, the oxygen consumed varies from 16.1 grams to 22.8 grams.

A second cause for the variation is the difference in rates of typewriting. Referring to table 12 it is seen that the average rates in the different experiments vary from 57 words per minute to 115 words per minute, so that if the normal metabolism for the individuals was the same, there would be a difference in the increase in metabolism due to the different rates in typewriting.

Finally, the variations may be due to a difference in efficiency, that is to say, the expenditure of energy by one individual in writing 1000 words may be greater than the energy expended by another.

Increase in metabolism per 1000 words.—Since there are marked variations in the percentage increase of metabolism during the typewriting periods, a more nearly uniform basis of comparison would be one in which the metabolism was compared on the basis of the same amount of writing. Accordingly the increase in metabolism per 1000 words for each of the different experiments has been calculated and presented in table 15. The three women subjects, A, B, and D, show remarkably uniform results per 1000 words, the carbon dioxide excretion only varying from 2.08 grams to 2.58 grams. Unfortunately the oxygen consumption was determined with only two of these subjects, and here the agreement is not quite so marked. The heat production for these three individuals varies from 4.17 to 6.08 calories per 1000 words. The two male subjects, C and E, show extremely wide variations, the results with subject C for all the factors being considerably larger than those with subject E, the latter showing very much lower results than those obtained with the three women subjects. The average increase per

TABLE 15.

Increase in metabolism per 1000 words and per 1000 strokes.

SUBJECT	DATE	STROKES PER WORD	CARBON DIOXIDE ELIMINATED		OXYGEN ABSORBED		HEAT PRODUCED	
			Per 1000 words	Per 1000 strokes	Per 1000 words	Per 1000 strokes	Per 1000 words	Per 1000 strokes
	1910		*grams*	*grams*	*grams*	*grams*	*cals.*	*cals.*
A..........	December 13..	5.0	2.58	0.50	2.28	0.44	6.50*	——
	December 28..	4.5	2.31	0.51	2.43	0.54	4.37	0.97
B..........	December 15..	6.0	2.35	0.41	2.57	0.44	5.07	0.88
	December 21..	5.5	2.15	0.40	1.87	0.35	4.17	0.78
C..........	December 27..	4.0	4.35	1.07	3.72	0.92	13.48	3.32
	December 29..	4.0	3.30	0.83	2.77	0.69	9.29	2.32
	1911							
D............	January 8.....	4.0	2.08	0.52	——	——	6.08	1.52
E............	January 15 ...	4.0	1.31	0.32	1.64	0.40	4.64	1.14
Average......		4.5	2.55	0.57	2.47	0.54	6.70	1.56

*Heat eliminated.

1000 words for all experiments with all individuals is 2.55 grams for carbon dioxide, 2.47 grams for oxygen, and 6.70 calories for the heat produced.

Increase in metabolism per 1000 strokes.—If a study is made of the column "Strokes per word," in table 15, it will be seen that in the three individuals who gave fairly uniform results on the basis of 1000 words, there is a marked variation in the number of strokes per word; and when the results are calculated on the basis of 1000 strokes, it is evident that the uniformity would not necessarily be so striking. In order to obtain a more accurate comparison, the results have been compared on the basis of per 1000 strokes. Here there is not so uniform an agreement in the factors of metabolism with the three individuals A, B, and D as was shown when the results were compared on the basis of per 1000 words, although the results for A and D are extremely close, *i.e.*, an average of 0.51 grams of carbon dioxide per 1000 strokes. The two experiments with subject A show a remarkably close agreement in the carbon dioxide excretion. In the two experiments with B, the carbon dioxide excretion per 1000 strokes also agrees very closely. Subject C shows a noticeable increase in metabolism per 1000 strokes, while subject E is again somewhat lower than the other subjects. The ratio of the differences between E and the women subjects, however, is not so great when considered on the basis of per 1000 strokes as when considered on the basis of per 1000 words. In the comparison per 1000 words, the heat production of E was slightly lower than that of any of the other subjects, with the exception of the heat production in the second experiments with subjects A and B, which fell a little below; in the comparison on the basis of 1000 strokes, however, the heat production with E is uniformly higher than with A and B. The average increase per 1000 strokes in all experiments with the 5 subjects is 0.57 grams of carbon dioxide, 0.54 grams of oygen, and 1.56 calories of heat produced. As shown previously, the average for the heat produced is low for the reason that the total heat production was not actually measured in all the experiments.

Increase in metabolism when writing at a uniform rate of 50 words per minute.—In all of these experiments the subjects attempted to write at a faster rate than they would normally in ordinary office work. They all considerd the experiments abnormal so far as the rate of writing was concerned as in their every day work they were not accustomed to writing at such speed. It is, therefore, of interest to note the increase in the various indices of total metabolism with the different individuals if calculated on the basis of the same rate of writing. In table 16 the results of such a calculation are given, showing the increase in metabolism per hour with a uniform rate of typewriting of 50 words per minute. The results given in this table were calculated in the following manner: At fifty words per minute a subject would write 3000 words per hour and therefore the data here presented are those given in table 15 multiplied by 3. This is on the assumption that the subjects would write words of the same length as those they wrote in the experiments. At a uniform rate of 50 words per minute, the increase in carbon dioxide excretion would range from 3.9 to 13.1 grams per hour, in oxygen consumption from 4.9 to 11.2 grams, and in heat production from 40.4 calories to 13.1 calories. These factors are also compared in this table upon the basis of the number of strokes, and the

TABLE 16.

Increase in metabolism when calculated to a rate of 50 words per minute and 13,900 strokes per hour.

(Amounts per hour).

SUBJECT	DATE	CARBON DIOXIDE ELIMINATED		OXYGEN ABSORBED		HEAT PRODUCED	
		Rate of 50 words per min.	Rate of 13,900 strokes per hour	Rate of 50 words per min.	Rate of 13,900 strokes per hour	Rate of 50 words per min.	Rate of 13,900 strokes per hour
	1910	*grams*	*grams*	*grams*	*grams*	*cals.*	*cals.*
A	December 13	7.7	7.0	6.8	6.1	—	—
	December 28	6.9	7.1	7.3	7.5	13.1	13.5
B	December 15	7.1	5.7	7.7	6.1	15.2	12.2
	December 21	6.5	5.6	5.6	4.9	12.5	10.8
C	December 27	13.1	14.9	11.2	12.8	40.4	46.1
	December 29	9.9	11.5	8.3	9.6	27.9	32.2
	1911						
D	January 8	6.2	7.2	—	—	18.2	21.1
E	January 15	3.9	4.4	4.9	5.6	13.9	15.8

increase in metabolism is shown when a subject is writing at the rate of 50 words of average length a minute or 4.6 strokes per word (13900 strokes per hour). This is calculated from the increase in metabolism per 1000 strokes, and multiplied by 13.9. The results agree very closely with those given in the previous table although they are somewhat more nearly uniform, the range in the increase of carbon dioxide being from 7.4 grams to 14.9 grams. If these individuals worked 8 hours a day at this rate, there would be an increase in energy output above resting varying from about 110 calories to 370 calories, the former figure in all probability being somewhat lower than the actual minimum with these individuals.

Variation in individuals.—In the tables thus far presented it has been seen that there is a marked variation among the individuals, regardless of the method of comparison. Subject C required from two to three times the amount of energy required by the three subjects A, B, and D for the same amount of work, while subject E expended even less energy than any of the three women subjects and only one-third of the requirement of subject C. The three women subjects evidently wrote with the same facility and with about the same expenditure of energy. This would indicate, therefore that among women the technique is more uniform than among men. On the other hand, of the two male subjects, C was out of training, not having operated a typewriter for some months previous, while E was paying particular attention to facility and was attending evening school with the special object of perfecting himself to the highest degree. The latter operated the typewriter upon the so-called "touch" system; so far as could be seen, he had in all probability a higher degree of technique and finish than any of the other four operators. Subject B said she also operated upon the "touch" system but it is to be questioned whether her technique was so perfect as that of subject E. Of the three women, however, the increase in metabolism per 1000 strokes is the least with subject B. With subject C, it was noticed that in operating the typewriter not only did he move his fingers and hands but also to some extent his whole body, particularly when writing fast. There was a rythmical backward and forward movement of the main portion of the body and this was noticed not only in practice but also in the actual experiments, where the head and shoulders could be observed. This would naturally result in a larger expenditure of energy, for if an individual in working uses not only the muscles required but makes other muscles work, the expenditure of energy is of course larger, other things being equal. It should also be stated that subject C had had part of one leg replaced by an artificial one. This, however, should in nowise affect his efficiency but would affect simply the base line on normal metabolism. An examination of the kymograph records shows that with this subject there were more marked irregularities in the respiration curves than with any other subject, and that the movements of the hands and arms in performing the work could be very easily detected from the kymograph record. The ranges in these experiments are due, it is believed, to technique and skill, rather than to individual variations in the machine or to other causes.

It is interesting, also, to compare the physical characteristics of the two men which show the extremes. Subject C was quite tall and at the same time somewhat stout. Subject E, having about the same height, was very much lighter in weight. Subjects A, B, nd D on the other hand were more nearly alike in physical con- our although subject D was somewhat heavier than the other two omen subjects. The variations are certainly not due to differ- nce in speed for the reason that the subjects A, B, and D wrote at idely varying speeds, subject A writing at the rate of 64 words er minute in one experiment and 80 in the other, subject B at the ate of 56 words in one experiment and 60 in the other, and subject at a much faster rate, *i.e.*, 115 words per minute.

Increase in metabolism in typewriting by periods.—Inasmuch as in everal of these experiments the metabolism was obtained not only or the rest and typewriting periods separately but also for type- riting by periods, there is an opportunity for considering the ques- ion of the influence of fatigue upon metabolism. The increase in etabolism per 1000 words and per 1000 strokes by periods is iven in table 17. Considering this table, it is seen that on the asis of per 1000 words there is in all cases, except C, a slowly iminishing increase in metabolism. This is most marked in the xperiment with subject E, in which the carbon dioxide excretion ncreased 1.60 grams, while in the last period, it increased but 0.99 ram. The oxygen determinations for the last two periods with sub- ect C were averaged together as the determinations for the individ- al periods were evidently in error. In the increase per 1000 trokes by periods, the differences are not so marked, although ith subjects A and E there is constantly in both carbon dioxide nd oxygen a diminution per 1000 strokes as the experiment con- inued. In neither instance, however, was the subject so fatigued s to be unable to continue. Subject E complained of having ramps in his hand during the last period which made it necessary or him to favor himself for a short time in order to recover. On he contrary, subject C, who shows very little, if any, diminution uring the 3 hours, was very much fatigued; in fact, he said he could ot have continued much longer and found it extremely difficult to eep up during the last hour. Subjects B and D do not show marked ifferences, while subjects A and E do. It is interesting to note hat both subjects A and E said that in the experiments their hands ecame cramped and tired, while subjects B and D both said that hey could continue longer without extra effort. The decreased nergy output of A and E as the experiment continued would seem o indicate that as they became fatigued, they worked with an increas-

TABLE 17.

Increase in metabolism by periods per 1000 words and per 1000 strokes.

SUBJECT	DATE	CARBON DIOXIDE ELIMINATED		OXYGEN ABSORBED	
		Per 1000 words	Per 1000 strokes	Per 1000 words	Per 1000 strokes
	1910	*grams*	*grams*	*grams*	*grams*
A	December 28	2.92	0.63	2.92	0.63
		2.04	0.45	2.36	0.52
		2.07	0.47	2.10	0.48
B	December 15	2.37	0.41	2.31	0.40
		2.32	0.40	2.84	0.49
	December 21	2.31	0.43	2.00	0.37
		1.99	0.37	1.73	0.32
C	December 29	3.39	0.85	2.43	0.61
		3.08	0.77	2.94	0.73
		3.45	0.86		
	1911				
D	January 8	2.21	0.55		
		2.22	0.55		
		1.79	0.45		
E	January 15	1.60	0.39	1.93	0.47
		1.36	0.33	1.34	0.33
		0.99	0.24	1.70	0.42

ing efficiency. This is contrary to the natural expectation, at least according to popular opinion, for it is generally supposed that as one becomes fatigued, the efficiency tends to decrease. In view of the fact that the metabolism of A and E decreased per 1000 strokes in the successive periods, it may be supposed that the fatigue tended to eliminate any extraneous muscular movements and that the subjects confined themselves to performing only those movements which were necessary to operate the typewriter to produce the result; also it may be pointed out tnat as the subjects became tired, while the number of words may not have diminished and the amount of typewriting was the same, the force with which it was done may have diminished, *i.e.*, at the beginning of the work, the subjects being fresh and well rested, struck the keys with more force than later on when they became more tired. There was no opportunity of gauging the amount of force with which the keys were struck in these experiments as the typewriting does not show any particular difference.

Respiratory quotient during rest and during typewriting. Inasmuch as the oxygen absorption and carbon dioxide elimination were obtained in these experiments in a fasting condition, there is an opportunity to compute the respiratory quotients for the individual experiments during rest and during typewriting. These are summarized as follows:

SUBJECT	RESPIRATORY QUOTIENT	
	Rest	Typewriting
A	84 84	83 78
B	85 78	78 79
C	85 82	85 85
E	88	79

It will be seen that in the first experiment with subject A, the second experiment with subject B, and the two experiments with subject C, there is a very close agreement in the respiratory quotients between rest and typewriting. This is of interest because of the fact that some authors have the idea that the respiratory quotient is raised during muscular work. In none of these experiments was the respiratory quotient raised and in some instances it was somewhat lower. It is believed by the writer, however, that a respiratory quotient in the typewriting periods lower than that in the rest periods indicates the probability of an error in the measurement of the oxygen absorption, this measurement for some reason being possibly too large.

In the later discussion of the equivalent amount of work calculated from the increase in the factors of metabolism, it will be seen that there are certain discrepancies in some of the experiments. In several instances, the amount of work calculated from the increase in oxygen is higher than that from the carbon dioxide. It so happens that in all the cases where there is a disagreement between the amount calculated from the carbon dioxide and that calculated from the oxygen, the respiratory quotient is lower in the typewriting period than in the rest period.

Calculation of the equivalent amount of work based upon the increase in the metabolism. It was impracticable to measure the actual amount of work performed in these experiments but other experiments in which the actual amount of work performed, the oxygen consumed, and the carbon dioxide, and heat produced were determined, have supplied data from which the equivalent amount of work in performing various operations can be calculated when the increase in carbon dioxide, oxygen, and heat are known. In the experiments referred to, the subject rode a bicycle in which the energy applied to the pedals was actually transformed into heat[1] and the factors have been calculated giving the increase in carbon dioxide eliminated, oxygen absorbed and heat produced per kilogrammeter.[2] Using these factors and the increases in carbon dioxide, oxygen, and heat in the typewriting experiments here reported, the amount of work done in these experiments has been calculated and is given in table 18. This table shows the total equivalent amount of work actually accomplished in the whole working period and the amount per hour. The calculations based upon the increase in carbon dioxide show a variation in work from 1773 to 4501 kilogrammeters. In some experiments the agreement between the amount of work as calculated from the three factors is remarkably close. In the experiment with subject A on December 13, the figures based upon the increase in carbon dioxide and oxygen were 2519, and 2544 kilogrammeters, respectively; the amount of work based on the heat elimination was 2215 kilogrammeters; in the second experiment with the same subject, however, the agree-

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[1] Benedict and Carpenter: U. S. Department of Agriculture, Office of Experiment Stations Bulletin No. 208, 1909.

[2] Benedict and Carpenter: *Carnegie Institution of Washington Publication No. 126,* 1910, p. 228.

TABLE 18.

Kilogrammeters of work calculated upon the increase of the various factors of metabolism.

SUBJECT	DATE	TIME	STROKES PER HOUR	UPON CARBON DIOXIDE ELIMINATED		UPON OXYGEN ABSORBED		UPON HEAT PRODUCED		AVERAGE PER HOUR	PER 1000 STROKES
				Total	Per hour	Total	Per hour	Total	Per hour		
	1910	*hours*									
A.........	December 13	2	20,173	5038	2519	5088	2544	4429*	2215†	2532	126
	December 28	2¼	21,700	5857	2834	7047	3410	3875	1875‡	3122	144
B.........	December 15	2	19,695	4015	2008	5029	2515	3027	1514‡	2262	115
	December 21	2	19,354	3913	1957	3889	1945	2652	1326‡	1951	101
C.........	December 27	3	16,606	13,504	4501	13,187	4396	14,598	4866	4588	276
	December 29	3	17,587	10,844	3615	10,380	3460	10,643	3548	3541	201
	1911										
D...........	January 8	2¼	27,606	7801	3467	——	——	7973	3544	3506	127
E...........	January 15	2¼	22,577	3990	1773	5731	2547	4938	2195	2172	96

* Calculated upon heat eliminated.
† Calculated upon heat eliminated; not included in the average.
‡ Not included in the average.

ment is not quite so close. In the second experiment with subject B, the work as calculated from the increases in the carbon dioxide and the oxygen agree remarkably. In the experiments with subject C, the increases based upon all three factors also agree fairly well, again indicating in all probability that the heat production of this subject was more accurately measured than in the experiments with the other subjects, with the possible exception of subject D. With this latter subject, the increases based upon carbon dioxide eliminated, oxygen absorbed, and heat produced also agree quite closely. It should be stated that in all of these experiments and all of these comparisons, it is considered that the carbon dioxide is the factor which is the most accurately measured. In experiments of this kind, in which the subject is without breakfast and has not eaten a heavy meal of carbohydrates the night before, it can be considered that the metabolism will consist of relatively the same proportion of carbohydrates, protein, and fat throughout the entire time of the experiment, *i.e.*, that the respiratory quotient for the early morning hours should not be materially different from that of the later hours of the morning or the first hours of the afternoon; consequently, the changes in carbon dioxide may be considered to represent the actual variations in metabolism. In some instances the variations shown in the oxygen consumed may also be considered a very good index of the changes in metabolism, particularly in experiment No. 1 with subject A, experiment No. 2 with subject B, and in the two experiments with subject C. In other experiments, *i.e.*, the second experiment with subject A and the experiment with subject E, in which the amount of work calculated upon the oxygen increase is greater than that calculated upon the carbon dioxide elimination, it must be assumed that the measure of the oxygen consumed is somewhat too large or else that there was an actual change in the character of the metabolism. If there were an actual change in the character of the metabolism, then the figures for the work done calculated upon the carbon dioxide would be too low. If the subjects wrote at a uniform rate of 50 words a minute and 13900 strokes an hour, the equivalent amount of work would vary from 1400 to 3900 kilogrammeters per hour.

Wolpert[1], using the increase in carbon dioxide as an index of the nt of work performed in various occupations, gives 1600 ammeters per hour for a tailor and 4100 kilogrammeters mechanic (apprentice). His factor was 300 kilogrammeters am of carbon dioxide while the factor used in these experi- is 257 kilogrammeters per gram of carbon dioxide increase, it the comparison is not on exactly the same basis, as his s would be somewhat higher than if the factor made use of se experiments were employed.

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[1] Wolpert: *Arch. f. Hygiene*, xxvi, p. 68, 1896.

nparison with earlier work. In table 19, a calculation has made of the increase per 1000 strokes in the two experiments ed in an earlier publication.[1] In comparing the results with 15, it will be seen that in both of these experiments the metab- per 1000 strokes based upon all three factors is somewhat than any of the results with the subjects here reported. In obability the facility and technique of these two individuals ot so high as those in the experiments here reported, as neither individuals reported upon in the earlier publication were tomed to typewriting continuously and, in fact, one of them een out of practice for some time.

TABLE 19.

se in metabolism per 1000 strokes in experiments with C. F. S. and W. C. A.

CT	DATE	NUMBER OF STROKES	CARBON DIOXIDE ELIMINATED		OXYGEN ABSORBED		HEAT PRODUCED	
			Total increase	Increase per 1000 strokes	Total increase	Increase per 1000 strokes	Total increase	Increase per 1000 strokes
	1905		*grams*		*grams*		*cals.*	
S....	October 24	17,728	28.4	1.60	25.8	1.46	59.2	3.34
A...	October 24	33,881	29.6	.87	30.3	.89	37.2	1.1C

SUMMARY.

pewriting at the rate of from 57 to 115 words per minute ed in an increase in the pulse rate from 90 to about 120, n the respiration per minute from 19 to 30.

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[1] rpenter and Benedict: This *Journal*, vi, p. 271, 1909.

There was also an increase in the body temperature, although it is evident that the total rise was not satisfactorily measured in these experiments.

The work of typewriting at the rates reported in these experiments resulted in an increase of 30 to 70 per cent in the total metabolism above the resting metabolism, or an average of about 50 per cent.

The amount of increased energy above the resting metabolism per 1000 words was on the average about 7 calories and the increase in both the carbon dioxide and the oxygen was 2.5 grams.

The increase in carbon dioxide and oxygen due to 1000 strokes upon a typewriter was 0.57 and 0.54 grams, respectively, and about 1.6 calories increase in energy output.

The measure of metabolism by periods during the work of typewriting shows that there was a gradual decrease in the amount of energy required per 1000 strokes. This was more marked in the individuals whose hands and fingers became extremely tired, which would indicate that during fatigue, work is accomplished more economically.

The average equivalent amount of work calculated from the increase in metabolism in these experiments varied from 1950 to 4600 kilogrammeters per hour and the calculation based upon these results gave an average of 145 kilogrammeters per 1000 strokes of typewriting.

The variation in the increase in metabolism due to the work of typewriting in these different individuals is considered to be due to differences in individual facility and technique. The female subjects showed a more uniform increase than the two male subjects who represented the extremes in these experiments.

PENTOSANS IN LOWER FUNGI.

By ARTHUR W. DOX and RAY E. NEIDIG.

(From the Chemical Section of the Iowa Agricultural Experiment Station.)

(Received for publication, March 13, 1911.)

It has long been known that many plant tissues when subjected to distillation with hydrochloric acid yield a volatile substance which passes over into the distillate, and can easily be identified as furfural. This is formed by simple dehydration and ring closure of the pentoses or five-carbon sugars. Inasmuch as pentoses are not found in the free state in nature, but are known to occur in combination in nucleoproteins and pentosans, we assume that the pentose from which the furfural is obtained by the above reaction, results from the hydrolysis of one or both of these substances.

Pentosans are of considerable importance to plant chemistry, both on account of their wide distribution and the comparatively large amounts in which they occur. They are an important constituent of the so-called wood gums which may be extracted from wood, straw, corncobs, bran, etc. Pentosan determinations are very commonly made in connection with the proximate analyses so important in the examination of vegetable foods and agricultural products. Very few determinations of this character have been made, however, upon the lower fungi, presumably because of the difficulty of obtaining pure cultures and cultivating the organisms on a sufficiently large scale to furnish material for analysis.

Wichers and Tollens[1] have made pentosan determinations upon some of the wood-destroying fungi. These investigators found a variation from 2.52 per cent to 6.73 per cent in the fifteen species examined, the average being about 4 per cent. The fungi studied in their experiments were organisms whose natural substratum contained pentosans and the possibility suggested itself that these might be the source of the pentosans contained in the mycelium of the fungus. A solution of this problem seemed to be offered in a parallel study of some of the Aspergillaceæ which thrive admirably in media containing hexoses as the only source of carbon. The presence of pentosans in cultures of this character would prove conclusively that the fungus cells have the power of constructing pentosans out of hexoses or their decomposition products.

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[1] *Journ. f. Landw.*, lviii, p. 238.

EXPERIMENTAL.

Six characteristic species of the common saprophytic molds were obtained in pure culture and inoculated upon a sterile medium consisting of cane sugar and inorganic salts. At the end of ten days the mycelium was separated from the culture fluid, carefully washed, ground and dried to constant weight at the temperature of boiling water. Five-gram samples of the resulting dry powder were distilled with 12 per cent hydrochloric acid, according to the provisional method of the Association of Official Agricultural Chemists, and the furfural that passed over was collected and weighed as the phloroglucide. The following table shows the amount obtained from each of the species examined.

SPECIES	PHLOROGLUCIDE	FURFURAL	PENTOSANS	PENTOSANS
	gram.	*gram.*	*gram.*	*per cent*
Aspergillus niger	0.0540	0.0307	0.0528	1.05
Aspergillus fumigatus	0.0492	0.0282	0.0486	0.97
Aspergillus clavatus	0.0452	0.0261	0.0450	0.90
Penicillium chrysogenum	0.0606	0.0341	0.0587	1.17
Penicillium camemberti	0.0496	0.0284	0.0489	0.97
Penicillium expansum	0.0431	0.0250	0.0432	0.86

The solution remaining after distilling off the furfural was treated with sodium acetate, then with sodium bisulphite and copper sulphate. Only a slight precipitate resulted, indicating that purines could not have been present in more than traces. Nucleoproteins, therefore, cannot be regarded as the source of the furfural obtained, and we must consider the latter as derived from pentosans. From these experiments it is evident that pentosans are normal constituents of the cell structure of lower fungi and are formed independently of the presence of pentose complexes in the culture medium. It is worthy of mention, however, that these lower fungi contain considerably less pentosan than the xylophagous fungi examined by Wichers and Tollens.

Owing to the difficulty of securing sufficiently large amounts of material, we did not attempt to isolate the pentosans and determine the exact nature of the pentose which they yield on hydrolysis.

SOME FACTORS INFLUENCING THE QUANTITATIVE DETERMINATION OF GLIADIN.[1]

By J. E. GREAVES.

(From the Chemical Laboratory of the Utah Experiment Station and the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, March 7, 1911.)

In testing the chemical composition of flours in work which is being carried on by a number of stations on the milling qualities of wheat, it is necessary to make each year a great number of gliadin determinations. The method[2] which has been used by the author gives comparative results if followed closely, but where the number of samples is large, the time required to make the determinations is too great; and the polariscope method given by Snyder[3] has not been applicable in most cases. Furthermore, no systematic work has been done to correlate the results obtained under varying conditions with these two methods. Therefore, this work is undertaken for two reason: first, to so modify the polariscope method that it may be used with flours from all wheats; and second, to find the relationship existing between the polariscope method and the Kjeldahl method, together with some of the factors which influence the results obtained by these methods.

The results reported in the following pages were obtained by the analysis of six different flours obtained from wheat of high, medium and low gluten content, grown on the Utah Experimental Arid Farms.

The extraction of the gliadin, unless otherwise stated, was made by placing a weighed portion of flour together with a measured amount of alcohol into 200 cc. wide-mouth bottles, provided with ground glass stoppers. By the use of bottles with ground stoppers the evaporation during extraction is reduced to a minimum and they furnish convenient receptacles in which the solution can be shaken at intervals during extraction. All the results reported are calculated to dry basis and are the average of three or more analyses which in nitrogen determinations agreed to within 0.025 per cent and the polariscope determinations within 0.1 on the sugar scale of a Schmidt and Haensch polariscope so that analytical errors have been reduced to a minimum.

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[1] Abstract of a thesis presented to the faculty of the University of California in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] *Utah Exp. Sta. Bull. No. 103.*

[3] *Journ. Amer. Chem. Soc.*, xxvi, p. 263, 1904.

PREPARATION OF A CLEAR FILTRATE.

One of the great objections which has been raised against the polariscope method for determining gliadin is the fact that the filtrate as obtained especially from high gluten flours, is cloudy, and for that reason the reading cannot be made with any degree of accuracy. If the suspended particles which cause the cloudy filtrate be insoluble proteins, as they sometimes are, an objection can be raised against the Kjeldahl method for determining gliadin as these will vitiate the results thus obtained. For this reason in both methods of determining gliadin a clear filtrate is of the utmost importance.

Thatcher[1] found it impossible to use the polariscope method on flour obtained from the Washington wheats. They were low in gluten and high in carbohydrates and gave cloudy filtrates. Snyder[2] found that soft wheats frequently gave a filtrate which was cloudy and became more cloudy on standing. The same difficulty was experienced by Shaw[3]. But the last two investigators were able to overcome this difficulty by decreasing the agitation of the samples during extraction.

In order to determine whether this method would give clear filtrates with the flours being examined, tests were made on the six samples of flour in which the shaking of the samples during extraction was gradually reduced to such an extent that the solutions could be filtered through double quantitative filters and thus give a clear filtrate. But it was found that by so doing the total nitrogen as determined by the Kjeldahl method had decreased and there was not the agreement between duplicates that was obtained when the samples were thoroughly shaken during extraction; thus showing that this means of obtaining a clear filtrate is not applicable with these flours.

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[1] *Journ. Amer. Chem. Soc.*, xxix, p. 910, 1907.
[2] *Ibid.*, xxvi, p. 263, 1904.
[3] *Ibid.*, xxix, p. 1747, 1907.

Teller[1] states that he obtained clear solutions of gliadin by filtering through a layer of animal charcoal, while Marion[2] in the polariscope method as outlined by him recommends the stirring of the solution at the end of the extraction period, with 0.8 gram of animal charcoal for from one to two minutes before filtering. Both of these methods were tried on the flours under examination and it was found that under these conditions clear filtrates could be obtained, but the animal charcoal in carrying down with it the suspended coloring material of the solution had also carried down some of the gliadin and this in varying amounts as was shown by the lack of agreement between duplicate determinations made by the Kjeldahl method. The method which was finally adopted after trying various others was to filter the solutions through asbestos. Filters of this were prepared as follows: a small amount of dry finely-shredded asbestos was placed in the bottom of a Gooch crucible and a pad of about one-half inch made by pouring suspended asbestos upon this. This was washed with alcohol, dried and the solution filtered through it. This gave a rapid method of filtering, and evaporation did not affect to any appreciable extent the concentration of the solution. Clear filtrates were obtained from all the flours by this method, and duplicates agreed very closely thus showing that the filter had no retarding influence on the nitrogenous substances. In order to reduce the color of the solutions, which was very dark from some of the flours, and in this manner to increase the accuracy of the polariscope readings, one-half the amount of flour recommended by Snyder was extracted with 100 cc. of alcohol (100 cc. of alcohol to 7.985 grams of flour in place of 15.97 grams). The effect of this upon the accuracy of the method in general will be considered later. Solutions prepared as above described and placed in well stoppered flasks may be kept for several hours without developing the cloudy appearance referred to by Thatcher and Snyder, providing the temperature does not fall to 10° C.

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[1] *Amer. Chem. Journ.*, xix, p. 59, 1897.

[2] *Ann. chim. analyt.*, xi, p. 134, 1906.

INFLUENCE OF RATIO OF ALCOHOL TO FLOUR ON GLIADIN EXTRACTED.

These determinations were made by extracting 15.97, 7.985, 3.9925, and 1.9962 grams of each of the flours with 100 cc. of 70 per cent alcohol by volume, and 15.97, 1.985 and 2 grams with 74 per cent alcohol for forty-eight hours with occasional thorough shaking. At the end of this time the solutions were filtered as described above and placed immediately into 200 cc. bottles provided with ground glass stoppers. Twenty cc. of this solution were used for nitrogen determinations by placing into Kjeldahl digestion flasks with 5 cc. of concentrated sulphuric acid and the alcohol distilled off. After this, 15 cc. more of concentrated sulphuric acid and 0.5 gram of mercuric oxide was added and the digestion completed. The total nitrogen was determined as outlined by the Association of Official Agricultural Chemists[1]. The remaining solution was used for polarization. In this a 200 mm. tube was used and an average taken of from six to eight readings. In table 1 is shown the per cent of nitrogen extracted by 70 and 74 per cent alcohol with varying proportions of flour to alcohol.

An examination of the table shows that the total alcohol-soluble protein is not obtained with either 70 or 74 per cent alcohol when 15.97 grams of flour are extracted with 100 cc. of alcohol even after the expiration of forty-eight hours, for in the above determinations the solvent was allowed to act for that length of time. It may be seen that the greater amount extracted by the alcohol when a small amount of flour was used, in proportion to the alcohol, is not a constant, but appears to vary with the flour and strength of alcohol, reaching the great difference of 0.113 per cent nitrogen with Black Don and only 0.013 per cent with Gold Coin. The average difference of the two strengths of alcohol, 70 and 74 per cent are nearly the same, being 0.051 and 0.061 per cent res-

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[1] *U. S. Dept. of Agr., Bureau of Chem., Bull.* No. 107 (Revised) p. 5.

TABLE 1.

Showing per cent of nitrogen extracted from flour by means of 70 and 74 per cent alcohol by volume with different ratios of alcohol to flour.

GRAMS OF FLOUR TO 100 CC. OF ALCOHOL	NEW ZEALAND	WHITE CLUB	GOLD COIN	BLACK DON	KAHLA	ADJINI	AVERAGE
100 cc. 70 per cent alcohol and 15.97 gm. flour	1.450	1.289	1.495	1.618	1.652	1.506	1.502
100 cc. 70 per cent alcohol and 7.985 gm. flour	1.470	1.333	1.508	1.731	1.675	1.592	1.552
Difference	0.020	0.044	0.013	0.113	0.023	0.086	0.050
100 cc. 70 per cent alcohol and 3.9926 gm. flour	1.544	1.397	1.539	1.701	1.767	1.616	1.593
Greater +, less −, than with charge of 7.985 gm. flour	0.074	0.064	0.031	−0.030	0.092	0.024	0.041
100 cc. 74 per cent alcohol and 1.9963 gm. flour	1.553	1.347	1.557	1.748	1.822	1.595	1.604
Greater than with charge of 7.985 gm.	0.083	0.014	0.049	0.017	0.147	0.003	0.052
100 cc. 74 per cent alcohol and 15.97 gm. flour	1.179	1.030	1.196	1.304	1.405	1.259	1.229
100 cc. 74 per cent alcohol and 7.985 gm. flour	1.278	1.106	1.291	1.329	1.448	1.289	1.290
Difference	0.099	0.076	0.095	0.025	0.043	0.030	0.061
100 cc. 74 per cent alcohol and 2 gm. flour	1.282	1.094	1.267	1.336	1.419	1.270	1.278
Greater +, less −, than with charge of 7.985	0.004	−0.008	−0.024	0.005	−0.029	−0.019	−.012

pectively. With the 70 per cent alcohol there is a greater amount of nitrogen obtained when the ratio is 3.9926 grams of flour to 100 cc. of alcohol and the amount is still greater when the ratio is 1.9963 grams of flour to 100 cc. of alcohol. Where 74 per cent alcohol was used as a solvent the nitrogen obtained is about the same whether 100 cc. of alcohol was used to extract 7.985 grams or 2 grams of flour. Where there is a difference it is within experimental error.

These results, especially as obtained with 70 per cent alcohol, are in keeping with those reported by Chamberlain[1] who found that in gliadin determinations relatively large amounts of solvent should be used with relatively small amounts of solute. He recommends that not over 2 grams of flour be used to 100 cc. of 70 per cent alcohol.

In table 2 is given the ratio of the per cent alcohol-soluble protein nitrogen as obtained by the Kjeldahl method divided by the polariscope reading on the sugar scale of a Schmidt and Haensch polariscope. The polarizations were made in 100, 200, and 400 mm. tubes with charges of 15.97, 7.985 and 3.9925 grams respectively.

TABLE 2.

Showing the ratio of per cent alcohol soluble nitrogen to polariscope reading with differing charges of flour in 70 per cent and 74 per cent alcohol by volume.

GRAMS OF FLOUR TO 100 CC. OF ALCOHOL	NEW ZEALAND	WHITE CLUB	GOLD COIN	BLACK DON	KAHLA	ADJINI	AVERAGE
15.97 gm. flour to 100 cc. 70 per cent alcohol	0.50	0.53	0.50	0.55	0.52	0.56	0.526
7.985 gm. flour to 100 cc. 70 per cent alcohol	0.51	0.53	0.50	0.56	0.51	0.54	0.525
3.9925 gm. of flour to 100 cc. 70 per cent alcohol	0.52	0.53	0.51	0.55	0.52	0.54	0.528
15.97 gm. flour to 100 cc. 74 per cent alcohol	0.48	0.44	0.45	0.47	0.46	0.48	0.463
7.985 gm. flour to 100 cc. 74 per cent alcohol	0.47	0.45	0.46	0.47	0.47	0.47	0.465

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[1] *Journ. Amer. Chem. Soc.*, xxviii, p. 1657, 1906.

An examination of these results shows that the ratio of alcohol-soluble protein to polariscope reading varies directly with the concentration of the solution. This is in accord with the results obtained by Mathewson[1] who found that the specific rotation of carefully prepared gliadin in 70 per cent alcohol is but little affected by ordinary changes in the gliadin concentration of the alcoholic extract. In the above results, however, the hard wheats show a slightly higher average ratio than do the soft wheats.

Inasmuch as the ratio of per cent nitrogen to polariscope reading is a constant[2] with varying ratios of alcohol to flour, and a greater amount of nitrogen is extracted when the ratio is 7.985 grams of flour to 100 cc. of alcohol than when it is 15.97 grams of flour to 100 cc. of alcohol, the former ratio has been used in the following work. That this does not decrease the accuracy of the polariscope method is shown by the following consideration. The dilute solutions are less colored than the more concentrated and for this reason the error in the polariscope reading is less in the dilute solution than in the more concentrated and darker ones. An error of 0.1 on the sugar scale of a Schmidt and Häensch polariscope (with solutions prepared as described above and polarized with care the error need never exceed this amount) would be equal to 0.046 per cent nitrogen in 74 per cent alcohol. While with the method as recommended by Snyder using the factor 0.2 as obtained by him there would be an error of 0.02 per cent nitrogen. This would have the result 0.026 per cent nitrogen in favor of the ratio 15.97 grams of flour to 100 cc. of alcohol. But as was shown above when this ratio is used the nitrogen extracted is 0.061 per cent less than when the ratio 7.985 grams of flour to 100 cc. of alcohol is used. This gives 0.035 per cent nitrogen in favor of the latter. With some flours which give fairly colorless solutions on extractions this error may be further reduced by using a 400 mm tube in the polarization. This, however, has not been found advisable with these high gluten flours as the darker color of the column of liquid decreases in too great a measure the accuracy of the polariscope reading.

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[1] *Journ. Amer. Chem. Soc.*, xxviii, p. 624, 1906.

[2] In this work within experimental error. That the specific rotation of a great number of organic compounds does vary with the concentration of the solution is a well established fact (*cf.* Landolt: *Rotation of Organic Substances*, Translated by Long, p. 169). That gliadin belongs to this class is quite likely from the work of Lindet and Aminann (*Ann. Inst. Nat. Ag. on.*, 1907, pp. 233-243). But from the work reported in these pages the difference would not appear to be of sufficient magnitude to materially effect determinations made by the polariscope method.

INFLUENCE OF DURATION OF EXTRACTION ON YIELD OF NITROGEN.

This was determined by extracting 7.985 grams of each of the flours with 70 and 74 per cent alcohol for 24 and 48 hours. The results obtained are given in table 3.

TABLE 3.

Showing per cent nitrogen extracted and ratio of polariscope reading to per cent nitrogen for each of the flours after extraction with 70 and 74 per cent alcohol for 24 and 48 hours.

DURATION OF EXTRACTION AND STRENGTH OF ALCOHOL	NEW ZEALAND	WHITE CLUB	GOLD COIN	BLACK DON	KAHLA	ADJINI	AVERAGE
70 per cent alcohol, 24 hours	1.450	1.289	1.505	1.721	1.732	1.558	1.543
74 per cent alcohol, 48 hours	1.470	1.333	1.508	1.731	1.735	1.592	1.562
Difference	0.020	0.044	0.003	0.010	0.003	0.034	0.019
Per cent nitrogen by polariscope reading 24 hours	0.50	0.53	0.50	0.56	0.54	0.57	0.533
Per cent nitrogen by polariscope reading 48 hours	0.51	0.53	0.50	0.56	0.51	0.54	0.525
74 per cent alcohol, 24 hours	1.270	1.086	1.293	1.320	1.446	1.278	1.282
74 per cent alcohol, 48 hours	1.278	1.106	1.291	1.329	1.448	1.289	1.290
Difference	0.008	0.020	−0.002	0.009	0.002	0.011	0.008

These results show that there is a small quantity of protein which passes into solution even after the expiration of 24 hours contact with the solvent. The increase is marked only in the case of the White Club and Adjini. With the remainder of the samples the increase after 24 hours is very small. That this increase is not due to evaporation is shown by the fact that the bottles containing the solution weighed the same at the beginning and end of the extraction period.

Examining the work which has been previously done on this subject, we find that Hummel[1] reports the amount extracted after the lapse of 44 hours to be slightly greater than that extracted at the end of 24 hours. Chamberlain[2] found the same to be true. He also compared the amount extracted at the end of 48 and 72 hours and found a small increase but it was not as great as in the preceding 24 hours. It is possible that the extra amount extracted after 24 hours is glutenin or some nitrogenous substance of the flour other than gliadin. However, the small difference in the chemical composition of gliadin and glutenin makes this rather difficult to determine.

That the specific rotation of the gliadin is not changed to any great extent by contact with 70 per cent alcohol for 48 hours seems probable from the results given, as the per cent nitrogen divided by polariscope reading is nearly constant for both periods. From these results it would appear that where as great accuracy as possible with our present methods is desired in the determination of the alcohol-soluble proteins, the extraction should be continued for 48 hours. But it must be borne in mind that when the length of the extraction period is increased, the error resulting from evaporation may be also increased if special precautions are not taken to prevent evaporation by the selection of bottles with well ground stoppers and the avoiding of excessive temperature during extraction. Nor should the flour be left in contact with the alcohol for too long a time, for if this be the case, part of the gliadin may become insoluble.

INFLUENCE OF STRENGTH OF ALCOHOL ON GLIADIN EXTRACTED.

Considerable work has been done to determine the effect of different strengths of alcohol on the extraction of gliadin from flour but in so far as I am aware, it has not been considered in connection with the rotation of the extract. And it is, primarily, in this connection, that it is to be considered in this article.

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[1] *U. S. Dept. of Agr. Bureau of Chem. Bull. 105*, p. 88, 1906.

[2] *U. S. Dept. of Agr. Bureau of Chem. Bull. 81*, p. 118, 1903.

Examining some of the work which has been previously done, on the extraction of gliadin from flour by varying strengths of alcohol, we find greatest amounts of nitrogen obtained with comparatively dilute alcohol. As for example Snyder[1] determined the nitrogen extracted by alcohol varying in strength from 60 to 72 per cent and found that the greatest amount, 85 per cent, was obtained with 60 per cent alcohol, and that the amount extracted decreased as the alcohol became more concentrated, the 72 per cent alcohol extracting only 0.67 per cent nitrogen. Hummel[2] determined the amount extracted with alcohol varying in strength from 70 to 81 per cent by weight, and found that 70 per cent alcohol had extracted 0.96 per cent nitrogen and that there was a gradual decrease in the yield to 75 per cent alcohol which extracted 0.66 per cent nitrogen, while the 81 per cent alcohol was found to extract only one-third as much as the 70 per cent. Shutt[3] tested alcohol ranging in concentration from 60 to 86.4 per cent by weight with similar results. While there is a greater amount of nitrogenous material extracted from the flour with the more dilute alcohol, there are facts which tend to show that the dilute alcohol also extracts a comparatively greater amount of non-gliadin nitrogen. This together with the fact that gliadin reaches its maximum solubility in 70 per cent alcohol has led a great number, though by no means all, investigators to adopt this as the proper strength of alcohol to use in gliadin determinations, a strength which appears from the following work to be too low.

The six different flours used in this work have been tested as to nitrogen extracted by varying concentrations of alcohol together with the influence on the polariscope reading as obtained with the alcoholic extract. This was determined by extracting 7.985 grams of each of the flours for 48 hours within 100 cc. of alcohol varying in strength from 60 to 80 per cent by volume. The results for the per cent nitrogen are given in table 4.

The maximium amount as may be seen, was extracted by 70 per cent alcohol. And with the exceptions of Black Don in which there is a large decrease, and Kahla a large increase, there is a gradual decrease to 60 per cent alcohol. As the strength of the alcohol is increased from 70 to 72.5 per cent there is a large decrease

[1] *U. S. Dept. Agr. Bureau of Chem. Bull.* 105, p. 88, 1906.
[2] *Ibid.*
[3] *Ibid.*

TABLE 4.

Showing the per cent of nitrogen extracted from flours from different wheats by varying strengths of alcohol.

STRENGTH OF ALCOHOL (PER CENT BY VOLUME)	NEW ZEALAND	WHITE CLUB	GOLD COIN	BLACK DON	KAHLA	ADJINI	AVERAGE
60 per cent	1.487	1.324	1.459	1.617	1.757	1.602	1.541
Greater +, less −, than by 70 per cent alcohol	0.017	−0.009	−0.049	−0.114	0.082	0.010	−0.063
65 per cent alcohol	1.429	1.267	1.467	1.644	1.666	1.499	1.496
Less than by 70 per cent alcohol	0.061	0.066	0.041	0.087	0.009	0.093	0.059
70 per cent	1.470	1.333	1.508	1.731	1.675	1.592	1.551
72.5 per cent	1.254	1.093	1.296	1.484	1.480	1.371	1.330
Less than by 70 per cent alcohol	0.216	0.240	0.212	0.247	0.195	0.221	0.221
74 per cent	1.278	1.106	1.291	1.329	1.448	1.289	1.290
Less than by 70 per cent alcohol	0.192	0.227	0.217	0.402	0.227	0.303	0.261
75 per cent	1.270	1.116	1.282	1.326	1.447	1.287	1.288
Less than by 70 per cent alcohol	0.200	0.217	0.226	0.405	0.228	0.305	0.263
80 per cent	1.118	0.997	1.192	1.288	1.441	1.249	1.214
Less than by 70 per cent alcohol	0.352	0.336	0.316	0.443	0.234	0.343	0.337

in the nitrogenous extract being as an average of these determinations 0.222 per cent nitrogen. As the strength of the alcohol is increased from 72.5 to 80 per cent there is a decrease in the nitrogen obtained but not in so marked a degree as between the 70 and 72.5 per cent alcohol. The proportion of the nitrogenous substances extracted by different strengths of alcohol, in some cases, appears to vary with the flour but in the main they all seem to follow the same trend.

The polariscope reading of solutions prepared by extracting flour with alcohol varying in strength from 60 to 80 per cent by volume was determined and are given in table 5 in the form of per cent nitrogen divided by the polariscope reading.

TABLE 5.

Showing the factor of per cent nitrogen as determined by the Kjeldahl method divided by the poloriscope reading for varying concentrations of alcohol.

STRENGTH OF ALCOHOL PER CENT BY VOLUME	NEW ZEALAND	WHITE CLUB	GOLD COIN	BLACK DON	KAHLA	ADJINI	AVERAGE
60 per cent alcohol	0.53	0.47	0.49	0.51	0.58	0.52	0.516
Greater +, less −, than by 70 per cent alcohol	0.02	−.06	−.01	−.05	.07	−.02	−.009
65 per cent alcohol	0.46	0.48	0.49	0.50	0.48	0.48	0.482
Less than by 70 per cent alcohol	0.05	0.05	0.01	0.06	0.03	0.06	0.043
70 per cent alcohol	0.51	0.53	0.50	0.56	0.51	0.54	0.525
72.5 per cent alcohol	0.47	0.44	0.45	0.51	0.51	0.48	0.477
Less than by 70 per cent alcohol	0.04	0.09	0.05	0.05	0.00	0.06	0.048
74 per cent alcohol	0.47	0.45	0.46	0.47	0.47	0.47	0.465
Less than by 70 per cent alcohol	0.04	0.08	0.04	0.09	0.04	0.07	0.060
75 per cent alcohol	0.47	0.45	0.47	0.46	0.48	0.48	0.468
Less than by 70 per cent alcohol	0.04	0.08	0.03	0.10	0.03	0.06	0.057
80 per cent alcohol	0.44	0.49	0.44	0.46	0.52	0.44	0.465
Less than by 70 per cent alcohol	0.07	0.04	0.06	0.10	0.01	0.10	0.060

An examination of the table 5 reveals the facts that with few exceptions the ratio of per cent nitrogen to polariscope reading is greatest where the flour has been extracted with 70 per cent alcohol, that this ratio decreases as the strength of alcohol increases from 70 to 80 per cent and that the difference between the ratios as shown by the different flours with the same strength of alcohol is least with 74 and 75 per cent alcohol. Examining the difference between the ratio as shown by the various samples extracted with 70 per cent alcohol, we find it to be 0.06, while with 74 per cent alcohol the difference is only 0.02. Comparing this latter result with the difference existing between various samples as extracted with other strengths of alcohol, except 75 per cent we find a still greater difference. This is very important, especially where gliadin is to be determined by the polarisation of the alcoholic extract and then multiplying the results thus obtained by a factor obtained from a series of determinations on different flours.

If the specific rotation for the dissolved substance, calculated as gliadin, be determined from the different percentages of nitrogen and corresponding polariscope readings for the various strengths of alcohol, the following results are obtained.

STRENGTH OF ALCOHOL PER CENT.	$[\alpha]_D$
60	−80.85°
65	−86.52°
70	−79.49°
72.5	−87.46
74	−89.80
75	−89.16
80	−89.71

These results show that the specific rotation reaches its highest value when the flour has been extracted by means of 74 per cent alcohol. This fact together with the nearly constant value obtained in nitrogen by polariscope reading with 74 per cent alcohol points very strongly to the conclusion that this strength of alcohol extracts more nearly pure gliadin than does any of the other strengths tested. However, the great difference in the amount of nitrogenous material extracted by 70 and 74 per cent alcohol raises the question as to whether the extraction of gliadin is as complete with 74 per cent as it is with 70 per cent alcohol. In order to throw some light upon this subject, the solubility of gliadin was determined in 70 and 74 per cent alcohol.

The solubility in 70 per cent alcohol of carefully prepared gliadin was found to be 0.0601 while in 74 per cent alcohol it was found to be 0.0538. The difference in the solubility in the two concentrations is very small so it would appear as if just as much gliadin would be extracted from 7.985 grams of flour with 100 cc. of 74 per cent alcohol as with the same amount of 70 per cent alcohol, for the gliadin in this weight of flour would not exceed 0.8 gram which is only one-seventh of the amount 100 cc. of 74 per cent alcohol is capable of dissolving.

With methods based on the determination of gliadin with alcohol as a solvent, we must try and find the strength of alcohol in which the non-gliadin material extracted reaches a minimum and still extract all of the gliadin. Quite definite conclusions can be drawn on this subject from a consideration of the results obtained for the specific rotation of the alcohol extracted protein, together with the solubility of gliadin. The specific rotation of the alcoholic extracted protein from wheat flour reaches its maximum in 74 per cent alcohol which indicates that the non-gliadin material reaches its minimum value in alcohol of this concentration; 100 cc. of 74 per cent alcohol is capable of dissolving 5.38 grams of gliadin and with the weight of flour (7.985 grams) used the amount to be extracted would not exceed 0.8 gram. And the fact that when one-third of this amount of flour was extracted with 100 cc. of 74 per cent alcohol the per cent of gliadin obtained was but little greater than when the full 7.985 grams was used, would make it appear as if the conditions outlined above were most nearly reached with 74 per cent alcohol.

INFLUENCE OF HOT EXTRACTION.

Kjeldahl[1] found that the temperature of the alcohol used in the extraction of the protein from wheat meal influenced but slightly the amount extracted. While Chamberlain[2] determined ount of protein in a sample of flour with cold and hot and obtained with the former 7.32 per cent protein and e later 7.47 per cent protein. The hot alcohol extracted tein than the cold, however it is difficult to state just rtion of this difference is due to the change in temperature olvent and what is due to the change in the concentration alcohol during extraction and the subsequent addition of to replace that lost by evaporation.

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[1] *Centralbl. Agr. Chem.*, xxv, p. 197, 1896.

[2] *Journ. Amer. Chem. Soc.*, xxviii, p. 1657, 1906.

on[1] apparently overcame this difficulty by extracting the a closed vessel with hot alcohol. In order to test this , the six flours used in this work were extracted in closed by placing 7.985 grams of each of the flours, together 0 cc. of 74 per cent alcohol into tightly stoppered pressure These were weighed and then heated in a water bath at r twenty-five minutes with occasional thorough shaking. hich they were cooled to 17°C, weighed, and the nitrogen lariscope determinations made as in the general method. ults together with those obtained by 48 hours, cold extrac- the flours with 74 per cent alcohol are given in table 6. ight of the flasks before and after extraction were practi- e same thus showing no appreciable amount of alcohol n lost by evaporation.

TABLE 6.

the per cent nitrogen extracted by hot and cold 74 per cent alcohol he ratio of per cent nitrogen to polariscope reading in each case.

IETY	PER CENT NITROGEN BY			RATIO OF PER CENT NITROGEN TO POLARISCOPE READING		
	74 Per cent cold alcohol	74 Per cent hot alcohol	Difference	74 Per cent cold alcohol	74 Per cent hot alcohol	Difference
land....	1.278	1.390	0.112	0.47	0.56	0.09
Club.....	1.106	1.165	0.059	0.45	0.52	0.07
in.......	1.291	1.393	0.102	0.46	0.59	0.13
on......	1.329	1.623	0.303	0.47	0.61	0.14
....	1.448	1.671	0.223	0.47	0.60	0.13
....	1.289	1.494	0.205	0.47	0.64	0.17
....	1.290	1.457	0.167	0.465	0.587	0.121

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chim. analyt., xi, p. 134, 1906.

These results show a greater per cent of protein nitrogen in every case where the extraction has been made with hot alcohol than where it has been made with cold. This difference varies with the different flours being greatest with Black Don and least with White Club. In fact all the Durum varieties show a larger difference than do the common bread varieties. However, in the case of all the flours there is a large difference between the hot and cold extraction and amounts to, as an average of three determinations, 0.167 per cent of nitrogen. The ratio of nitrogen to polariscope reading is also higher in the hot extraction and there is a lack of agreement in this ratio with the different flours when the hot alcohol is used as the solvent.

The solutions obtained by cooling and filtering after hot extraction were clear, but on standing for a few hours they became turbid and in a short time a fine precipitate settled out. This was filtered off and the filtrate allowed to stand for 24 hours during which time there again developed a turbid solution. The fact that alcoholic solutions of gliadin and solutions made by cold extraction of flour may be kept for several days without becoming turbid, indicates that the gliadin was either changed during the hot extraction or that some substance other than gliadin was extracted by the hot alcohol, which on standing, slowly separated out. That it is due to the latter cause is likely from the fact that gliadin solutions gave the same specific rotation after heating for twenty-five minutes at 65°C. as they did before.

INFLUENCE OF HEATING FLOUR BEFORE EXTRACTION.

This was determined by heating 7.985 grams of each sample for 16 hours in a steam bath at 96°C. They were then extracted with 74 per cent alcohol for 48 hours, and the nitrogen and polariscope determinations made as in the preceding work. The results together with those obtained with the air-dry flour are given in table 7.

As may be seen, there is a variation with the different flours nevertheless, with the single exception of Black Don, the air-dry flour on extraction yields considerably more protein than does the flour which has been heated at 98°C. for 16 hours. This difference being as an average of all the determinations 0.085 per cent nitrogen. This is not as great a difference as was obtained by Chamberlain[1] who has studied the action of the alcoholic extraction of dry and air dry flour with the result that he obtained 4.58 per cent protein from a sample of dry flour and 7.32 per cent from the sample in the air-dry condition.

The ratio of per cent nitrogen to polariscope reading, within experimental error, is the same under both conditions. It is likely that the portion which has been rendered insoluble is mainly albumen as very little of the globulin would be coagulated at the temperature used in drying the flour. That it is not the gliadin is likely from the work of Mathewson who has shown that gliadin apparently suffers no change when heated at the above temperature for sixteen hours. However, the conditions for gliadin are different when heated in the air-dry flour than when the nearly pure gliadin is heated, so that too much reliance must not be put upon his work. The very nearly agreeing ratios of nitrogen to polariscope reading in the case of heated and non-heated flour show that if the gliadin be not rendered insoluble by the heating, the substance which is rendered insoluble, has very nearly the same specific rotation as that of gliadin. Furthermore, there was a very

TABLE 7.

Showing the per cent nitrogen extracted by 74 per cent alcohol from dry and air-dry flour, together with the ratio of per cent nitrogen to polariscope reading in each case.

VARIETY	PER CENT NITROGEN EXTRACTED			PER CENT NITROGEN BY POLARISCOPE READING		
	From dry flour	From air-dry flour	Difference	Dry flour	Air-dry flour	Difference
New Zealand....	1.118	1.278	0.160	0.46	0.47	0.01
White Club.....	1.016	1.106	0.090	0.44	0.45	0.01
Gold Coin.......	1.187	1.291	0.104	0.43	0.46	0.03
Black Don......	1.317	1.329	0.012	0.46	0.47	0.01
Kahla...........	1.365	1.448	0.083	0.48	0.47	0.01
Adjini...........	1.225	1.289	0.064	0.48	0.47	0.01
Average.........	1.205	1.290	0.085	0.459	0.465	0.006

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[1] *Journ. Amer. Chem. Soc.*, xxviii, p. 1657, 1906.

close agreement between duplicate determinations a condition which would not be expected if part of the gliadin had been rendered insoluble.

EXTRACTION WITH ETHER AND THEN ALCOHOL.

This determination was made to ascertain the effect of the ether-soluble substances (such as lecithin, which contains nitrogen and is optically active) on the results obtained by the Kjeldahl and polariscope methods. The determinations were made by extracting 7.985 grams of each of the flours with "anhydrous ether" in a Soxlet extraction apparatus for eight hours, drying at a low temperature long enough to dispell all the ether, extracting for forty-eight hours with 74 per cent alcohol and determining the nitrogen and polariscope readings as in the preceding work. The results are given in table 8, together with those obtained by the direct extraction of air-dry flour with 74 per cent alcohol.

TABLE 8.

Showing the per cent nitrogen extracted by 74 per cent alcohol from ether-extracted and air-dry flour; also the ratio of per cent nitrogen to polariscope reading in each case.

VARIETY	PER CENT NITROGEN EXTRACTED			RATIO OF PER CENT NITROGEN TO POLARISCOPE READING		
	From ether extracted flour	From air-dry flour	Difference	For ether extracted flour	For air-dry flour	Difference
New Zealand....	1.142	1.278	0.136	0.45	0.47	0.02
White Club.....	1.987	1.106	0.119	0.47	0.45	−0.02
Gold Coin.......	1.229	1.291	0.062	0.44	0.46	0.02
Black Don......	1.323	1.329	0.006	0.50	0.47	−0.03
Kahla...........	1.442	1.448	0.006	0.50	0.47	−0.03
Adjini...........	1.278	1.289	0.011	0.49	0.47	−0.02
Average.........	1.234	1.290	0.056	0.475	0.465	0.01

An examination of the above table shows that in the case of the common bread varieties of flour, there is a much greater per cent of nitrogen extracted from the air-dry flour than from flour which has been previously extracted with ether. However, with the flours from the Durum wheats the results are, within experimental error, the same for both extractions. The ratio of per cent nitrogen to polariscope reading shows a small difference in the two determinations but there is no regularity in the difference. The solutions which were obtained from the ether extracted flour were nearly colorless while some of those obtained by the direct extraction of the flour with alcohol, although clear, were quite highly colored. This was greatest in the case of Black Don and Kahla and least with New Zealand and Gold Coin.

In order to determine to what extent the gliadin had been dissolved by the ether, two-gram portions of carefully prepared gliadin were extracted with ether in a Soxlet extraction apparatus for four hours, the ether evaporated and the resulting residue taken up with 100 cc. of 74 per cent alcohol. The rotation of this solution was practically zero. The nitrogen in the solution was determined by the Kjeldahl method and it was found to contain 0.9 mg. of nitrogen. The gliadin which had been used in the first extraction was again extracted with ether for four hours and the nitrogen determined as before with the result that the ether had extracted 0.14 mg. of nitrogen. The results show that the gliadin is dissolved only very slowly by the ether and taken in connection with the results obtained from the extraction of the flour with ether, they show that some flours contain sufficient ether soluble nitrogen carrying substance to materially affect gliadin determinations as made by the direct extraction of air dry flour with 74 per cent alcohol.

INFLUENCE OF TEMPERATURE ON THE POLARISCOPE READING.

Solutions were prepared as in the general method and the polariscope readings taken at different temperatures. It was found that solutions filtered at room temperature (about 17°C.) on being cooled to below 10°C. gave a turbid solution and for this reason the first reading was taken at 10°C. and subsequently readings at 20, 30, 40, 50 and 60°C. The average of all the results obtained from all the flours was found to be 0.15 more on the sugar scale of a Schmidt and Haensch polariscope at 10°C. than at 60°C. This would correspond to a difference of 0.003 on the sugar scale for a change of each degree in temperature. Therefore, a rise of 10°C.

which in ordinary work is far beyond the change in temperature that would be likely to occur is equal to 0.03 on the sugar scale. This would correspond to only 0.012 per cent nitrogen which is within experimental error. For this reason, it is not necessary to make a correction for the differences in temperature. However, it is best when working with the polariscope to make all readings at between 15° and 20°C. for above this temperature evaporation is comparatively great, and below this temperature the solution, if filtered at 15°C. tends to become turbid, thus preventing an exact reading.

THE INFLUENCE OF NON-PROTEIN SUBSTANCES ON THE POLARISCOPE READING.

Some analysts have found it necessary when determining gliadin, by means of the polariscope method, to make a correction for the sugars extracted with the gliadin, while others have found this to be unnecessary.[1]

Determinations have therefore been made on the six different flours used in this work to find out the effect of non-protein substances on the results obtained by means of the polariscope. This was done by precipitating the protein material from 50 cc. of the solution prepared as in the preceding determinations by means of 5 cc. of a saturated solution of mercuric nitrate, and then polarizing. The correction thus obtained, in terms of per cent on the sugar scale of a Schmidt and Haensch polariscope, was found to be as an average of all the flours 0.223. The lowest reading was obtained for Gold Coin 0.159 and the highest for Adjini 0.281. The results were found to be the same, within experimental error, when the flours had been extracted with cold 74 per cent alcohol, in the air-dry condition, after extraction with ether after heating 16 hours and when extracted with hot 74 per cent alcohol.

These results show that with flours similar to those used in this work it is necessary to make two polarizations so as to correct for non-protein material which has been extracted by the alcohol. Nor is it sufficient to make an average correction for in the six flours used there was found a difference of 0.122 on the sugar scale of a Schmidt and Haensch polariscope, between Gold Coin and Adjini.

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[1] Snyder: *Journ. Amer. Chem. Soc.*, xxvi, p. 263, 1904; Norton: *Ibid.*, xxvii, p. 922, 1905; Shaw: *Ibid.*, xxix, p. 1747, 1907; Ladd: *U. S. Dept. of Agric. Bureau of Chem. Bull. No. 122*, p. 53; Marion: *Ann. chim. analyt.*, xi, p. 134, 1906.

It is interesting to compare the average results obtained for per cent nitrogen, corrected ratio of per cent nitrogen to polariscope reading, and calculated specific rotation for the various extractions with 74 per cent alcohol. These results are given below.

	AIR-DRY FLOUR	HEATED FLOUR	LESS THAN AIR-DRY	ETHER EXTRACTED FLOUR	GREATER (+) OR LESS THAN (−) AIR-DRY	HOT EXTRACTION	GREATER (+), LESS (−) THAN AIR-DRY
er cent nitrogen..	1.290	1.205	−0.085	1.234	−0.056	1.457	+0.167
atio of per cent nitrogen to polariscope reading....	0.430	0.423	−0.007	0.438	+0.008	0.536	−0.106
alculated specific rotation.........	−97.01°	−98.61°		−95.30°		−77.86°	

These results show that when flour is extracted with hot 74 per cent alcohol in a closed vessel a greater per cent of nitrogen is obtained than when the flour is extracted with cold alcohol of the same strength. However, an examination of the calculated specific rotations show that the hot alcohol extract considerable non-gliadin protein. The nitrogen extracted by cold 74 per cent alcohol from flour which had been previously extracted with ether, or had been heated before extraction, is less than that extracted from the air-dry flour.

The ratio of per cent nitrogen to polariscope reading is nearly the same for air-dry flour, heated flour, and ether-extracted flour, but is much higher for flour extracted with hot alcohol. The specific rotation is highest for heated flour and lowest for flour extracted with hot alcohol.

SUMMARY.

The concentration of solutions of the alcohol-soluble protein is decreased on filtering through layers of animal charcoal or on being clarified by shaking with this substance and then filtering.

Solutions of alcohol-soluble proteins can be filtered through carefully prepared asbestos filters and in this manner clear filtrates obtained without materially changing the concentration of the solution.

As an average of the determinations made, 0.05 per cent more alcohol-soluble protein nitrogen was extracted when 7.985 grams of flour were treated with 100 cc. of alcohol than when twice this amount of flour was used with the same volume of alcohol.

The extraction of the alcohol-soluble proteins does not seem to be complete, especially with 70 per cent alcohol, when the proportion of flour to alcohol exceeds 2 grams of flour to 100 cc. of alcohol.

The specific rotation of alcohol-soluble proteins varies but little if any with the concentration of the solution.

Greater accuracy can be obtained in making gliadin determinations by means of the polariscope when 7.985 grams of flour are extracted with 100 cc. of alcohol and polarized in a 200 mm. tube than when twice this amount of flour is used with the same volume of alcohol. However, with flours from some wheats the accuracy of the method can be increased still more by extracting 7.985 grams of flour with 100 cc. of alcohol and then polarizing in a 400 mm. tube.

The amount of protein nitrogen extracted from flour varies with the strength of alcohol used. And with the strengths tested, 60 to 80 per cent, the greatest amount was extracted by 65 per cent alcohol by volume and there was a decrease in this amount as the strength of the alcohol increased.

A consideration of the specific rotation of proteins extracted by alcohol of varying strengths shows that alcohol of 74 per cent by volume more nearly extracts pure gliadin than does alcohol of other strengths.

From a consideration of the solubility of gliadin and the amount extracted with different ratios of alcohol to flour it appears that with a charge of 7.985 grams of flour to 100 cc. of alcohol, 74 per cent alcohol extracts as much gliadin as does 70 per cent alcohol.

The ratio of per cent nitrogen extracted from flour by alcohol to the polariscope reading for the solution varies with the strength of the alcohol..

The extraction of flour with hot 74 per cent alcohol in a closed vessel yields more protein nitrogen than does cold extraction. However, the specific rotation of the proteins extracted shows it to contain considerable non-gliadin protein material.

The heating of flour before extraction with alcohol decreases the amount of protein nitrogen extracted by 74 per cent alcohol.

Some flours contain sufficient ether-soluble nitrogen carrying substances to materially affect the accuracy of gliadin determinations made by the direct extraction of flour with 74 per cent alcohol.

The rotation of alcoholic extracts of flour is only slightly affected by changes in temperature and may, within certain limits, be disregarded in determining gliadin by means of the polariscope.

With the flours examined it was found necessary to make a correction for the sugars in the polariscope method.

Gliadin determinations can be made rapidly by means of the polariscope and the results thus obtained are fairly accurate, but not as accurate as those obtained by the Kjeldahl method.

ON THE COMPOSITION OF CERTAIN SUBSTANCES PRODUCED BY THE ACTION OF PEPSIN UPON THE PRODUCTS OF THE COMPLETE PEPTIC HYDROLYSIS OF CASEIN.

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When neutral or faintly acid solutions of caseinates of bases are acted upon by small quantities of pepsin at 30°-36° a variable quantity of a white precipitate, protein in nature and rich in phosphorus, is produced to which the term paranuclein has been applied.[1] In previous communications it has been shown by one of us[2] that when this substance is subjected to partial digestion by weak alkali a product results, which resembles paranuclein very closely in its properties and solubilities, but differs from it in the possession of a considerably lower phosphorus content; to this substance the provisional term "Paranuclein A" has been applied. It has further been shown that if concentrated solutions of pepsin be allowed to act at 36° upon the concentrated products of the complete hydrolysis of casein a substance, closely resembling Paranuclein A in its properties,[3] phosphorus content, and solubilities, is gradually deposited from the mixture, and that a very similar substance is produced more quickly if concentrated solutions of pepsin be made to act upon *unconcentrated* solutions of the products of the complete hydrolysis of casein at a considerably higher temperature, namely 65°, which is between 10° and 15° above the temperature at which the *hydrolytic* activity of pepsin is stated to be destroyed.

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[1] Kossel: Verh. d. Berl. physiol. Ges., *Arch. f.* (*Anat. und*) *Physiol.*, 1891, p. 181. For literature consult Gustav Mann: *Chemistry of the Proteids*, 1906, pp. 395-396.

[2] T. Brailsford Robertson: This *Journal*, iii, p. 95, 1907; v, p. 493, 1909.

[3] T. Brailsford Robertson: This *Journal*, viii, p. 287, 1910.

With a view to further elucidating the relationship between these substances we have determined their carbon, hydrogen and nitrogen content, with the following results.

(1) *Paranuclein, Obtained by the Partial Hydrolysis of Casein.*

To 8000 cc. of $\frac{N}{50}$ KOH which had been rendered just acid to resolic acid by the addition of casein (about 40 grams per liter) were added 4 grams of Grübler's pepsin puriss. sicc. which had previously been dissolved in a little distilled water. After the addition of excess of toluol (to render and keep the mixture sterile) and thorough agitation the mixture was kept at 36° for 6 days. At the end of that time a thick white precipitate had formed and remained suspended within the fluid. The mixture was then heated to 100° in a steam sterilizer for one-half hour, cooled and allowed to stand at 36° for 24 hours. At the end of that time a thick white precipitate had settled to the bottom of the container. This was filtered off, washed in a large volume of distilled water and set aside under alcohol for 5 months. To this was then added the similar precipitate which was obtained by treating 6 liters of $\frac{N}{50}$ KOH containing about 4 per cent of casein with pepsin for a week (adding 2 grams every two days) and similarly prepared. After a few days the supernatant alcohol was poured off from the combined precipitates. They were then suspended in 8 liters of distilled water and sufficient NaOH was added to render the solution tenth-normal. The entire precipitate dissolved readily, forming a clear yellowish solution. To this after rapid filtration through glass wool, were added 60 cc. of glacial acetic acid. The thick white precipitate which was obtained was washed with 10 liters of distilled water, agitated thoroughly and allowed to settle in tall glass cylinders. The supernatant water was then removed by decantation and this process was repeated six times. The precipitate was then washed in 8 liters of Kahlbaum's 99.8 per cent alcohol in two successive washings. It was the collected on a hardened filter paper and washed with about 4 kilos of Kahlbaum's ether (über Natrium destilliert). Finally, it was placed in a mortar and triturated with 1 kilo of ether; the supernatant ether was poured off and the precipitate was dried at 30° over calcium chloride and then at room temperatures over sulphuric acid. The paranuclein was thus obtained in the form of a fine, light, friable, white powder.

0.1771 gm. substance gave 0.3246 gm. CO_2 and 0.1146 gm. H_2O.

0.1 gm. substance gave 10.8 ccm. nitrogen at 11° and 750 mm. pressure.

	Found:
C	49.98 per cent
H	7.20 per cent
N	12.80 per cent

These figures agree very closely with those obtained by Lubavin.[1] It will be observed that the percentage composition of paranuclein differs from that of the majority of animal proteins in that the carbon content is somewhat and the nitrogen content considerably lower than that which is characteristic of the majority of proteins, including casein.[2] It also differs very strikingly from the "plasteins" and "coaguloses" which are obtained by the coagulating or rennet-like action of *dilute* pepsin or of insoluble powders upon the mixed and concentrated products of the *incomplete* peptic hydrolysis of proteins,[3] since these bodies are especially characterized by their abnormally *high* carbon content.[4]

(2) *Paranuclein A, Obtained by the Action of Lime-Water upon Paranuclein.*

It has been found by one of us[5] that if paranuclein, prepared in the manner described above and containing 4.2 per cent of P_2O_5 be digested in excess of calcium hydrate, a substance of lower phosphorus content (1.6 per cent P_2O_5) and somewhat less soluble in acids is produced. This substance has been provisionally termed Paranuclein A. If digestion with calcium hydrate be continued still further, or if it be carried out at a higher temperature, all substances precipitable by acetic acid disappear from the solution (*e.g.*, digestion for 18 hours at 34° of a 0.25 per cent solution of paranuclein in saturated calcium hydrate). It therefore appears possible that in successive hydrolytic cleavages the paranuclein which is first produced in a peptic digest successively loses phosphorus which is split off as phosphoric acid[1] until, finally, stance or substances are produced which are no longer in in neutral or faintly acid solutions, *i.e.*, which have lost one characteristic properties of the paranuclein group.

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[1] Lubavin: *Hoppe-Seyler's Med-chem. Untersuch.*, Berlin, 1866, p. 463.

[2] Cf. Hammarsten: *Text-book of Physiological Chemistry*, Trans. by Mandel, New York, 1904, pp. 27 and 440.

[3] Kühne and Chittenden: *Zeitschr. f. Biol.*, xix, p. 159, 1883. Okunew: Dissertation, St. Petersburg, cited after Maly's *Jahresber. f. Tierchem.*, 1895, p. 291: Lawrow: *Zeitschr. f. physiol. Chem.*, li, p. 1, 1907; liii, p. 1, 1907; lvi, p. 343, 1908. Sawjalow: *Arch. f. d. ges. Physiol.*, lxxxv, p. 171, 1901; *Zeitschr. f. physiol. Chem.*, liv, p. 119, 1907. Lawrow and Salaskin: *Zeitschr. f. physiol. Chem.*, xxxvi, p. 277, 1902; Kurajeff: *Beitr. z. chem. Physiol. und Path.*, i, p. 121, 1901; iv, p. 476, 1904; Umber: *Zeitschr. f. physiol. Chem.*, xxv, p. 258, 1898.

[4] Kühne and Chittenden: *loc. cit.* etc.

[5] T. Brailsford Robertson: This *Journal*, iii, p. 95, 1907.

Paranuclein A was prepared in the following manner.

Ten grams of the paranuclein described above were dissolve liters of saturated calcium hydrate and the mixture, in the pre excess of toluol, was allowed to stand at room temperature for 1 Seventeen cc. of glacial acetic acid were then added and an abund culant precipitate resulted, part of which settled to the bottom of t tainer, the remainder floating to the top. On agitating the mixt allowing it to settle again the whole of the precipitate sank to the l This was washed several times, by decantation, with water and th alcohol (99.8 per cent, Kahlbaum). The precipitate was collected on ened filter paper, washed with five liters of 99.8 per cent alcohol and of ether (Kahlbaum's über Natrium destilliert) and dried at 30° o cium chloride and then at room-temperature over sulphuric acid. nuclein A was thus obtained in the form of a light greyish-brown po cakes which were easily pulverized. The estimated quantity of the was 4 grams.

0.2127 gm. substance gave 0.3858 gm. CO_2 and 0.1305 gm. H_2O.

0.2 gm. substance gave 21.0 cc. nitrogen at 11.5° and 757 mm. p

	Found:
C	49.47 per cent
H	6.80 per cent
N	12.50 per cent

It will be observed that in its carbon, hydrogen, and ni content Paranuclein A is practically indistinguishable from nuclein.

(3) *The Substance which is Synthesised through the Ac Concentrated Pepsin at 36° upon the Concentrated P of the Complete Peptic Hydrolysis of Casein.*

It has been shown[2] that if the filtered products of the co peptic hydrolysis of an approximately 4 per cent solution of in $\frac{N}{50}$ sodium or potassium hydroxide be concentrated five times and 30 cc. of a 10 per cent solution of Grübler's pepsin puriss. sicc. be added to 70 cc. of the concentrated solution thus obtained, after a lapse of from 2 to 48 hours a white precipitate is formed within the mixture, which, when collected and purified, is found to very closely resemble Paranuclein A in its properties and phosphorus content. In the publications alluded to above evidence has been brought forward tending to show that in this case we have a real systhesis of protein from the products of its hydrolytic cleavage, and the endeavor has been made to interpret the mechanism which accomplishes this synthesis.

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[1] Salkowski and Hahn: *Arch. f. d. ges. Physiol.*, lix, p. 225, 1895.

[2] T. Brailsford Robertson: This *Journal*, iii, p. 95, 1907; v, p. 49 viii, p. 287, 1910.

Eighteen hundred cc. of the filtered products of the complete peptic hydrolysis of a 4 per cent solution of casein in $\frac{N}{50}$ sodium hydrate, free from substances precipitable by acetic acid, either with or without the previous addition of alkali, were evaporated to 300 cc. To this solution were added 130 cc. of 10 per cent pepsin (Grübler's puriss. sicc.) and the mixture was set aside at 36° in the presence of excess of toluol. Within 24 hours a precipitate had appeared in the fluid. After 5 days the precipitate was collected on a filter and washed with water until the washings were colorlesss. About 200 cc. of water containing 30 cc. of $\frac{N}{10}$ sodium hydrate were then poured into the filter and the contents agitated while the drippings were caught in about an equal volume of water containing 75 cc. of $\frac{N}{10}$ acetic acid. The resultant precipitate was collected on a filter, washed with water, 2 liters of 99.8 per cent alcohol and one liter of ether (U. N. D.) and dried at 36° over calcium chloride and then at room-temperature over sulphuric acid. The substance thus obtained was a friable white powder very faintly tinged with yellow.

0.1796 gm. substance gave 0.3684 gm. CO_2 and 0.1227 gm. H_2O.

0.2 gm. substance gave 21.9 cc. nitrogen at 11.5° and 758 mm. pressure.

	Found:
C	55.90 per cent
H	7.60 per cent
N	13.00 per cent

The nitrogen and hydrogen contents correspond tolerably with those of paranuclein and Paranuclein A but the carbon content is markedly higher. This forcibly suggested that the toluol employed in keeping the digest sterile might have been dragged down by the slowly settling precipitate. Accordingly another preparation was made.

Forty-two hundred cc. of the filtered products of the complete peptic hydrolysis of a 4 per cent solution of casein in $\frac{N}{50}$ sodium hydrate, free from substances precipitable by acetic acid, were evaporated to 600 cc. To [illegible] cc. of this solution were added 135 cc. of 10 per cent pepsin and the mixture was set aside at 36° in the presence of only a few drops of toluol. Within 15 hours a precipitate had appeared in the fluid. After only 48 hours the precipitate was collected and purified as described above, the reprecipitation being repeated twice, however, larger volumes of alcohol and ether employed in washing and toluol excluded during the operations of purification. This preparation yielded the following figures:

0.1400 gm. substance gave 0.2773 gm. CO_2 and 0.094 gm. H_2O.

	Found:
C................	54.0 per cent
H................	7.5 per cent

The carbon, it will be observed, was slightly lower than in the previous preparation. This encouraged the idea that the high carbon was due to associated toluol, and an endeavor was thereupon made to find a sterilizing agent, for employment during the digestion of the casein, which does not contain the aromatic group. No satisfactory substitute for toluol could be found, however. Accordingly a third preparation was made in which toluol was rigidly excluded throughout the process except during the initial digestion of the casein. Chloroform was employed to keep the concentrated mixture of products and pepsin sterile. This product yielded the following figures:—

0.1220 gm. substance gave 0.2384 gm. CO_2 and 0.0858 gm. H_2O.

	Found:
C................	53.39 per cent
H................	7.80 per cent

The carbon being again lower, but still high. On the whole, having regard to the similarity of its general chemical behavior and physical properties to those of Paranuclein A and to the fact that the synthetic substance which is obtained at higher temperatures (see below) is identical in its carbon, hydrogen and nitrogen content, with Paranuclein A, it appears probable that the two substances are in reality identical; but that the synthetic substance prepared in the manner described above, is associated with some contamination which contains a greater percentage of carbon, possibly the toluol employed in sterilizing the digest, or, more probably a protein, possibly a coagulose.

4) *The Substance which is Synthesised through the Action of Concentrated Pepsin at 60° upon the Unconcentrated Products of the Complete Peptic Hydrolysis of Casein.*

It has been shown by one of us[1] that it is possible to synthesise a substance apparently identical, in physical properties and chemical behavior, with the above, and closely resembling Paranuclein A, from the *unconcentrated* solution of the products of the complete peptic hydrolysis of a 4 per cent solution of casein in $\frac{N}{10}$ sodium or potassium hydroxide, provided the synthesis be carried out at a high temperature (60° to 70°) and in the presence of considerable excess of pepsin. These temperatures are from 10° to 15° in excess of the temperature at which, according to the majority of observers, pepsin is rapidly and completely deprived of its proteolytic activity.[2] From this and from other evidence pointing in the same direction, it has been argued that the synthesis which occurs in these solutions is not an example of true "reversion" of a catalysed reaction, but that it is due to a shift in the equilibrium between the protein and its products consequent upon a shift in the equilibrium between two forms of the enzyme, one of which accelerates the hydrolysis and the other the synthesis of the protein.

To 1500 cc. of the filtered and unconcentrated products of the complete peptic hydrolysis of 4 per cent casein dissolved in $\frac{N}{10}$ sodium hydrate, free from substances precipitable by acetic acid, were added 300 cc. of 15 per cent pepsin (Grübler's puriss. sicc.) and excess of toluol, both solutions having previously been heated to 60°. The mixture was then set aside at 60°-65°. Within three hours a heavy precipitate had appeared within the mixture. After 48 hours the precipitate was collected on a filter, and thereafter this substance was prepared in exactly the same manner as the substance described above. The product which is thus obtained is a light greyish-white friable powder.

0.3898 gm. substance gave 0.7145 gm. CO_2 and 0.2474 gm. H_2O.

Nitrogen was determined by the Kjeldahl method 0.5 gm. of substance being employed.

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[1] T. Brailsford Robertson: This *Journal*, v, p. 493, 1909; viii, p. 287, 1910.

[2] Cf. Oppenheimer: *Ferments and their Actions*, trans. by Ainsworth Mitchell, London, p. 92, 1901; A. E. Taylor; On Fermentation, *Univ. of Calif. Publ. Pathol.*, i, p. 253, 1907; Schwarz: *Beitr. z. chem. Physiol. und Path.*, vi, p. 524, 1905.

	Found:
C	49.99 per cent
H	7.00 per cent
N	13.10 per cent

This substance therefore, so far as its content of carbon, hydrogen and nitrogen is concerned, is indistinguishable from paranuclein.

SUMMARY.

1. The carbon, hydrogen, and nitrogen contents of paranuclein, Paranuclein A and the synthetic substances produced by the action of concentrated pepsin upon the products of the complete peptic hydrolysis of casein have been determined.
2. Paranuclein and Paranuclein A are indistinguishable from one another in their content of carbon, hydrogen and nitrogen. They differ from the majority of proteins, and very markedly from the coaguloses and plasteins by their low content of carbon.
3. The synthetic product obtained by the action of concentrated pepsin at 60° to 70° upon the *unconcentrated* products of the complete peptic hydrolysis of casein is identical, in its carbon, hydrogen and nitrogen content, with paranuclein and with Paranuclein A.
4. The synthetic product obtained by the action of concentrated pepsin at 36° upon the *concentrated* products of the complete peptic hydrolysis of casein differs, in three preparations examined, from paranuclein in its somewhat higher carbon content. Reasons are advanced in favor of the belief that this is attributable to contamination of the product, incident upon its method of preparation.

CONTRIBUTIONS TO THE THEORY OF THE MODE OF ACTION OF INORGANIC SALTS UPON PROTEINS IN SOLUTION.

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It was pointed out by Hardy[1] in his exhaustive communication on globulins, that the precipitation of proteins and, indeed, of colloids in general, may be of two kinds. The first is clearly accompanied by decomposition of the precipitating agent: it will not occur, as Pauli has demonstrated,[2] unless the protein is in some proportion ionic, and relatively small quantities of the precipitating agent are required to bring about the precipitation. The second kind of precipitation however, whether accompanied by decomposition of the precipitating agent or not (and data on this head are lacking), occurs even when the protein is non-ionic and requires relatively large amounts of the precipitating agent. Precipitation of the first kind is, generally speaking, only brought about by electrolytes, while precipitation of the second kind, although, as a rule, more readily brought about by electrolytes than by non-electrolytes, may nevertheless be brought about by non-electrolytes, for example by alcohol.

For this latter type of precipitation we shall henceforth, whenever possible, reserve the term *coagulation*.[3]

Both precipitation and coagulation of a protein may be brought about by one and the same inorganic salt. In such a case the gradual addition of a salt to the original salt-free solution which contains ionic protein (*i.e.*, protein which drifts to one electrode or to the other in an electric field) first brings about precipitation and then resolution of the protein. In this new solution the protein is, according to Hardy, non-ionic and can be coagulated by still further addition of the salt.

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[1] W. B. Hardy: *Journ. of Physiol.*, xxxiii, p. 251, 1905.

[2] W. Pauli: Hofmeister's *Beiträge*, vii, p. 531, 1906.

[3] Much confusion exists in the literature of this subject on account of the fact that the distinction between the *precipitation* of a protein through chemical interaction with the added salt, and its *coagulation* through the change in the nature of the solvent resulting from the further addition of salt, has not invariably been recognized.

The first kind of precipitation appears to be undoubtedly chemical in character and mechanism. The active agent in bringing about the precipitation of electro-negative colloids, *i.e.*, of colloids which migrate to the anode, is the cation of the added salt, that in bringing about the precipitation of electro-positive colloids is the anion.[1]

Protein behaves in acid solution like a cation and anions render it insoluble. In alkaline solution it behaves like an anion, migrating to the anode, and cations render it insoluble. The rate of precipitation is proportional, other things being equal, to the molecular conductivity of the added salt, *i.e.*, to the active mass of the precipitating ion.[2] The precipitation of colloids by small quantities of salts is accompanied by decomposition of the precipitating agent,[3] the precipitating ion being bound by the colloid and carried down with it, different ions being bound and replaceable by one another in equivalent proportions. The valency of the precipitating ion is, as is well known, of great importance in determining the rate of precipitation, the velocity of precipitation by mono- di- and trivalent ions respectively being in the proportion $1:x:x^2$.

The mechanism of *coagulation* is far from clear. The investigations of Hofmeister[4] and of Pauli,[5] however, have shown that

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[1] Hans Schultze: *Journ. f. prakt. Chem.*, xxv, p. 431, 1882; xxvi, p. 320, 1883; Prost: *Bull. Acad. Roy. Sci. de Belg.*, (3) xiv, p. 312, 1887; S. E. Linder and H. Picton: *Chem. Journ.*, lxi, p. 137, 1892; W. B. Hardy, *Proc. Roy. Soc.*, London, lxvi, p. 110, 1900.

[2] Hardy: *loc. cit.*

[3] Linder and Picton: *loc. cit.* Whitney and Ober: *Zeitschr. f. physikal. Chem.*, xxxix, p. 630, 1902.

[4] Hofmeister: *Zeitschr. f. anal. Chem.*, xx, p. 319; *Arch. f. exper. Path. u. Pharm.*, xxiv, p. 247, 1888; xxv, p. 1, 1888; xxvii, p. 395, 1890; xxviii, p. 210, 1891. Kauder: *Ibid.*, xx, p. 411, 1886; Pohl: *Ibid.*, xx, p. 426, 1886; Lewith: *Ibid.*, xxiv, p. 1, 1888.

[5] Pauli: *Arch. f. d. ges. Physiol.* lxxi, p. 333, 1898; lxxviii, p. 315, 1899; *Beitr. z. chem. Physiol. u. Path.*, iii, p. 225, 1903; v, 27, 1904; vi, p. 233, 1905;

dehydration of the dissolved protein plays an extremely important part in bringing about this phenomenon. The salt appears to coagulate the protein by competing with it for water, in other words by diminishing the active mass of the solvent. It has been shown by Pauli that the solvent or coagulating action of salts upon protein is an additive effect of their ions. The cations *induce* the coagulation of electro-negative protein (*i.e.*, of protein which behaves electrically like an acid[1]) in the following order, the most effective being placed first, the least effective last:

$$\mathrm{Li} > \mathrm{Na} > \mathrm{K} > \mathrm{NH_4} > \mathrm{Mg}$$

while anions *inhibit* the coagulation of electronegative protein in the order:—

$$\mathrm{CNS} > \mathrm{I} > \mathrm{Br} > \mathrm{NO_3} > \mathrm{Cl} > \text{Acetate} > \mathrm{SO_4} > \mathrm{F}$$

and the order of efficacy of the different cations in bringing about the coagulation of electro-negative protein is the order of the efficacy in diminishing the solubility of phenylthiocarbamid.[2]

For electro-positive protein, as Posternak[3] first showed, the series is exactly reversed. Those ions which coagulate the electro-negative protein most energetically most strongly inhibit the coagulation of electro-positive protein, while those which most strongly inhibit the coagulation of electro-negative protein are the most powerful precipitants of electro-positive protein.

Hofmeister (1890-1891) showed that the power of inorganic salts to inhibit the taking up of water by plates of gelatin runs parallel with their power to coagulate solutions of gelatin; while Pauli showed that those salts which coagulate gelatin most powerfully in strong solutions, in weaker solutions lower the tem-

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vii, p. 531, 1906. Pauli and Rona: *Biochem. Zeitschr.* ii, p. 1, 1902; Pauli and Samec; *Ibid.*, xvii, p. 235, 1909; Pauli and Handovski: *Biochem. Zeitschr.*, xviii, p. 340, 1909; xxiv, p. 239, 1910; Pauli and Wagner: *Ibid.*, xxvii, p. 296, 1910.

[1] J. Loeb: *Univ. of Calif. Publ., Physiol.*, i, p. 149, 1904.

[2] Rothmund: *Zeitschr. f. physik. Chem.*, xxxiii, p. 401, 1900; Rothmund and Wilsmore: *Ibid.*, xl, p. 611, 1902; Euler: *Ibid.*, xxxi, p. 360; Pauli: *Beitr. z. chem. Physiol. und Path.*, iii, p. 225, 1903.

[3] Posternak: *Ann d. l'Institut Pasteur*, xv, pp. 85, 169, 451, 570, 1901; Cf. also Pauli *loc. cit.* and Höber: *Beitr z. chem. Physiol. und Path.*, xi, p. 35, 1907.

perature of gelatinization while those salts which coagulate egg-globulin most powerfully also lower the temperature of heat-coagulation most markedly. A very striking proof of the fact that the solution of proteins by water is accompanied by a binding of water by the protein is that afforded by the investigations of Pauli and Semec who have shown that the presence of proteins in water considerably lowers the solubility of difficultly soluble inorganic salts.

The influence of valency upon the *coagulating* power of ions would appear to be much less pronounced than its influence upon their *precipitating* power, since magnesium resembles the alkalies closely in its coagulative power, while lithium approaches the alkaline earths.

The influence of added salts of the alkalies and magnesium upon the precipitation of proteins by heavy metals varies with the concentration of salt employed (Pauli). At low concentrations (0.005M) the salts inhibit precipitation in the order:

$$SO_4 < Cl < C_2H_3O < NO_3 < Br < I < SCN$$

while in high concentrations (4M) they *encourage* precipitation (or coagulation) in the order:

$$SO_4 > Cl > C_2H_3O > NO_3 > Br > I > SCN$$

This is simply a particular instance of the general rule that the salts may act as precipitants and as coagulants at low and at high concentrations respectively, acting as solvents at intermediate concentrations. The heavy metal salts afford no exception to this rule. At low concentrations they precipitate, at higher concentrations they dissolve and at still higher concentrations they coagulate the proteins of egg-white (Pauli) The concentration-range throughout which the salt acts as solvent may be evanescent however, as it is in the case of silver nitrate acting upon egg-albumin.

The very important observation has been made by Pauli[1] that absolutely electrolyte-free egg-albumin is not ionic (*i.e.*, does not drift in an electric field) and that under these conditions it is not precipitable by heavy metals. It is, however, *coagulable* by highly concentrated salts.[1]

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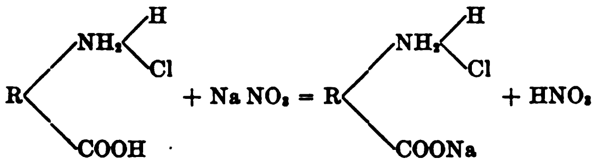
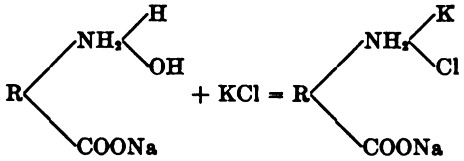
[1] Pauli: *Beitr. z. chem. Physiol. und Path.*, vii, p. 531, 1906.

According to Pauli and Handovsky[2] the number of ionized protein particles in a solution of ionic protein is diminished by the addition of salts; at the same time the viscosity of the solution diminishes and the coagulability of the protein by the usual coagulating agents is increased. When salts are added to a solution of electro-positive protein (*i.e.*, protein combined with acid) an increase in the acidity of the solution results, but when they are added to a solution of electro-negative protein (*i.e.*, protein combined with alkali) no increase in the alkalinity of the solution can be detected.[3] They believe that acid-protein reacts with salts as follows:



while alkali-protein reacts as follows:



This hypothesis it will be observed, involves the assumption that the proteins combine with acids and bases through the agency of terminal $-NH_2$ and $-COOH$ groups. The results of experiments which I have carried out during the past two years,[4] however, on the electrochemistry of the protein salts have convinced me that this, the commonly accepted view of the mode of combination of proteins with acids and bases, must be abandoned and that the combination of proteins with inorganic acids and bases is, in reality, accomplished in quite a different manner. This necessarily involves a corresponding modification of Pauli's hypothesis.

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[1] Egg-albumin is nearly equally basic and acid. It is possible, indeed, probable that pronouncedly acid or basic proteins, if soluble at all in the free condition, are also ionized.

[2] *Loc. cit.*, also Pauli and Wagner: *loc. cit.*

[3] Hardy: *Journ. of Physiol.*, xxxiii, 1905.

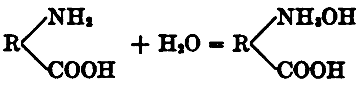
[4] Studies in the Electrochemistry of the Proteins, *Journ. of Physical Chem.*, 1909–1911.

THE MODE OF FORMATION AND IONIZATION OF THE COMPOUNDS OF PROTEINS WITH INORGANIC ACIDS AND BASES.

The investigations of Emil Fischer and of Kossel have shown us that the proteins are built up from amino-acids linked up in a catenary manner, and have demonstrated that the mode of linkage is expressed by the formula, -COHN-.

By methods which are too well-known to require description here, Fischer has succeeded in building up chains of amino-acids linked together in this manner which he terms "polypeptids," many of which exhibit characteristic properties of the peptones, such as precipitability by ammonium sulphate, digestibility by trypsin, etc., and some of which have been found to occur in incomplete protein digests as intermediate products of hydrolysis.

These polypeptids and, presumably, the proteins, are as essentially amino-acids as the amino-acids out of which they are built up. Thus glycyl-glycin, $NH_2.CH_2CO.NH.CH_2.COOH$, is as typically an amino-acid as glycocoll itself ($NH_2.CH_2.COOH$), since it possesses an $-NH_2$ group as well as a -COOH group, and hence is capable of forming compounds both with acids and with bases. On undergoing electrolytic dissociation it may be supposed to yield either hydrogen (H^+) ions, or hydroxyl ions, owing to the occurrence of a reaction with water of the type:—



$$R\left\langle\begin{matrix}NH_2\\COOH\end{matrix}\right. + H_2O = R\left\langle\begin{matrix}NH_3OH\\COOH\end{matrix}\right.$$

just as ammonia, in solution, partially reacts with water to form NH_4OH.

It is usually conceded that these elements in the structure of the proteins afford an explanation of the power which they possess neutralizing both acids and bases; the "amphoteric" character the proteins. To this opinion I have also formerly inclined, but accumulation of data irreconcilable with this view have induced e to abandon it. The weight of evidence appears irresistible at some elements in the protein molecule other than terminal H_2 or –COOH groups are responsible for the acid- and base-utralizing power which is possessed in such a marked degree many proteins.

In the first place the investigations of Levites[1] have shown that ly a very small proportion of the nitrogen in proteins is present thin their molecules in the form of $-NH_2$ groups, and even his imates of the $-NH_2$ nitrogen are probably in excess of the true lues.[2] Now edestin, as Osborne has shown,[3] is insoluble, when the free condition, in water. It forms an insoluble hydroloride containing 14×10^{-5} equivalents of HCl per gram and, further addition of acid, passes into solution. Its combining pacity for acids does not remain constant, however, for at neutral-to tropaeolin, which corresponds[4] to a reaction of from 0.01 0.001 N H^+, it neutralizes 127×10^{-5} equivalents of acid per am. Hence, if the acid is neutralized by the $-NH_2$ groups of estin, the number of these groups[5] must be at least $\frac{127}{14} = 9$. om the former determination, it would appear that the molelar weight of edestin is 7000, and this corresponds with the lecular weight indicated by its tyrosin and glutamic acid con-t[6] (= I mol. tryosin + 3 mols. glutamic acid + ...). Nine $-NH_2$ oups in this molecule would correspond to over ten per cent of the total nitrogen.[1] From the investigations of Erb,[2] although the exact interpretation which is to be placed upon his results is not perfectly clear, it would appear that the combining-weight of egg-albumin for acids may be as low as 152, while its molecular weight is, according to Hofmeister, 5400 or some multiple of this. Hence, upon the assumption that terminal $-NH_2$ groups bind the acids, there must be at least 35 of them in egg-albumin,[3] which would correspond to no less than 69 per cent of the total nitrogen in this protein.

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[1] Levites: *Zeitschr. f. physiol. Chem.*, xliii, p. 202, 1904; *Biochem. Zeitschr.*, p. 224, 1909.

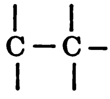
[2] Emil Fischer: *Untersuchungen über Aminosäuren, Polypeptide und Prone*, Berlin, 1906, p. 52.

[3] T. B. Osborne: *Journ. Amer. Chem. Soc.*, xxi, p. 486, 1899; *Zeitschr. f. ysiol. Chem.*, xxxiii, p. 240, 1901.

[4] Salm: *Zeitschr. f. physikal. Chem.*, lvii, p. 471, 1907.

[5] Osborne believes that an insoluble "monochlorhydrate,' is also formed, taining 7×10^{-5} equivalents of acid per gram, which would raise this mber to 18, but would, at the same time, double the estimate of the molelar weight.

[6] Kossel and Patten: *Zeitschr. f. physiol. Chem.*, xxxviii, p. 39, 1903.

The number of terminal $-COOH$ groups cannot be much in excess of the number of terminal $-NH_2$ groups, since the protein would otherwise be overwhelmingly acid in character[4] and the majority of the proteins possess a distinct capacity for neutralizing acids, even when they are themselves predominantly acid. Now free casein[5] is insoluble in water, but when combined with acids or with bases it is soluble. To carry 1 gram of casein into solution 11.4×10^{-5} equivalents of base just suffice, indicating a molecular weight, for the casein, of about 8800 or a multiple of this. The tyrosin, glutamic acid and sulphur contents of casein indicate a minimal molecular weight of from 4000 to 4400. In the presence of *excess* of base, however, casein attains a maximal combining-capacity (measured by the gas-chain) of 180×10^{-5} equivalents per gram, so that it behaves like a 16-basic acid, and if $-COOH$ groups bind the base there must be 16 of them in the molecule, corresponding to 25 per cent of the total oxygen or, almost exactly, *to the number of –COOH groups contained in the glutamic acid radicals in the casein molecule, calculated for the free acid.* In order to provide so many free carboxyls the form of the casein molecule would have to be that of a branched chain, or the radiating spokes of a wheel, at the centre of which must exist unions of the type:  $-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-$ and the regular decomposition of this protein into its constituent amino-acids, upon hydrolysis, would be unintelligible. Moreover, in the synthetical polypeptids, which closely resemble the natural peptones in their behavior, the linkage of the amino-acids is not radial but catenary in character[1] and the peptids which have been isolated from the mixed products of partial protein hydrolysis are likewise catenary in structure.

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[1] Or almost exactly to the $-NH_2$ content of the arginin, calculated as the *free* diamino-acid, which the edestin molecule contains. Kossel (*Zeitschr. f. physiol. Chem.*, xxv, p. 165, 1898) attributes the basic properties of proteins to their content of hexone base, and to this view electrochemical data lend very decided support. Edestin contains 11.7 per cent of arginin, but only insignificant amounts (1.0 per cent and 1.1 per cent respectively) of lysin and histidin. As at least one of the $-NH_2$ groups of the arginin is almost certainly present in the protein molecule in the *imino* form, it is highly improbable that the acid is neutralised by terminal *amino* groups supplied by the arginin.

[2] Erb: *Zeitschr. f. Biol.*, xli, p. 309, 1901.

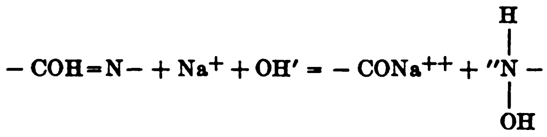
[3] Gustav Mann: *Chemistry of the Proteids*, London, 1906, p. 147.

[4] Hofmeister: *Ergeb. d. Physiol.*, I, Abt. I, 1902.

[5] T. Brailsford Robertson: *Journ. of Physical Chem.*, xiii, p. 469, 1909; xiv, p. 528, 1910, etc.

To account for the high acid- and base-combining capacity of the proteins we must therefore look to some other point in the molecule than terminal $-NH_2$ or –COOH groups. The point of union –C–N–C– immediately suggests itself. The type of this union which occurs in the polypeptids and, presumably, in the proteins is either –CO.NH– or –C(OH) = N– between which, the keto– and the –enol forms, synthetical data do not suffice to decide.[2]

According to Werner's theory of valencies the nitrogen in either of these unions contains two latent valencies, positive and negative, which, while the nitrogen is trivalent, neutralize one another internally, but which, when the nitrogen becomes pentavalent, are capable, respectively of neutralizing a negative or a positive radical. The second of the above types of union (the –enol form) carries with it the possibility of the following types of reactions



$$-\mathrm{COH}=\mathrm{N}- + \mathrm{Na}^{+} + \mathrm{OH}' = -\mathrm{CONa}^{++} + {}''\overset{\displaystyle \mathrm{H} \atop |}{\underset{\displaystyle | \atop \mathrm{OH}}{\mathrm{N}}}-$$

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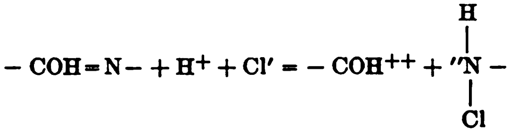
[1] Even when dicarboxylic acids enter into the compound. Cf. Emil Fischer and Ernst Koenigs: *Ber. d. d. chem. Ges.*, xxxvii, p. 4585, 1904; Emil Fischer and Julius Schmidlin: *Annalen der Chem.*, cccxl, p. 123, 1905.

[2] Cf. Aders Plimmer: *The Chemical Constitution of the Proteins*, London, 1908, part 2.

and:—



$$-\mathrm{COH}{=}\mathrm{N}- + \mathrm{H}^{+} + \mathrm{Cl}' = -\mathrm{COH}^{++} + {}''\overset{\overset{\mathrm{H}}{|}}{\underset{\underset{\mathrm{Cl}}{|}}{\mathrm{N}}}-$$

yielding, in each case, only *protein* ions.[1]

In a communication which will appear shortly[2] it will be shown in greater detail that this hypothesis regarding the mode of union between the proteins and bases and acids is supported by, among others the following facts:—

(1) The compounds which the proteins form with acids and bases, when dissolved in water, are excellent conductors of electricity and true electrolytes.[3] yet they do not yield chlorine ions when, for example, the compound in question is a hydrochloric acid compound,[4] nor do the potassium hydrate compounds yield potassium ions, or the calcium hydrate compounds calcium ions.[5] The equivalent conductivities of the compounds at infinite dilutions are such as would indicate the presence of *bulky organic ions,* travelling under a unit fall of potential at the constant min-

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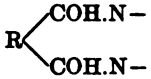
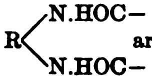
[1] Electrochemical data show that, in reality, dicarboxylic acid groups of the type  are the active agents in neutralizing bases, while diamino-acid groups of the type  are the active agents in neutralizing acids. Both $-\mathrm{COH.N}-$ groups of the carboxylic acid or diamino-acid radical must therefore be opened up before ionisation can occur. The above formulae should therefore be doubled. But since this fact does not essentially affect the application of this hypothesis to the precipitation and coagulation of proteins by salts, for simplicity of representation I only depict one of the two $-\mathrm{COHN}-$ groups which are actually involved.

[2] Studies in the Electrochemistry of the Proteins, Part 7, *Journ. of Physical Chem.*, 1911.

[3] Cf. Sjöquist: *Skand. Arch. f. Physiol.*, v, p. 277, 1894; T. Brailsford Robertson: *Journ. of Physical Chem.*, xi, p. 542, 1907; xii, p. 473, 1908; xiv, p. 528, 1910 etc. also Billitzer: *Ann. d. Physik.*, cccxvi, pp. 902, 937, 1903.

[4] Bugarszky and Liebermann: *Arch. f. d. ges. Physiol.*, lxxii, p. 51, 1898.

[5] T. Brailsford Robertson: *loc. cit.*

mal equivalent velocity[1] of 20×10^{-5} cm. per sec. at 30° which is characteristic of such ions.[2]

(2). Edestin will displace NaOH from its combination with hydrochloric acid[3] and casein, although insoluble in water, will displace carbonic acid from its combination with calcium hydrate.[4] Solutions of the caseinates of the bases may be obtained which are pronouncedly acid in reaction[5] and which do not alter in composition, *i.e.*, undergo hydrolytic dissociation, upon dilution. Since in all these cases the *molecular* concentration of the proteins is very low, and the compounds which are formed are quite highly electrolytically dissociated, were the formation of these compounds due to the replacement of OH' groups in $-NH_3OH$ groups by acid radicals or of H^+ groups in $-COOH$ groups by bases, then the "strength," *i.e.*, degree of dissociation of edestin as a base must be greater than that of NaOH, while that of casein as an acid must be much greater than H_2CO_3 and comparable with the degree of dissociation of NaOH at very high dilution. Such conclusions, applied to bodies which are amphoteric are, of course, absurd. Were the formation of potassium caseinate due to the formation of a salt such as $R\text{-}COO' + K^+$, an acid solution of this compound could no more exist than an acid solution of potassium aluminate. As in similar cases which occur in the domain of inorganic chemistry, we can interpret these phenomena only by assuming that the basic radical in the casein compound and the acid radical in the edestin compound are bound up in a non-dissociable form. Since the casein compounds, at least, when in solution in water, are notable conductors of electricity, they must dissociate at some other point in the molecule than that of the union between the base and the protein. The fact that the composition of protein salts is independent of the total dilution of the system and dependent only upon the relative masses of protein and acid or base *is attributable to the fact that water does not enter into either of the equations given above.*

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[1] That is, velocity per unit valency.

[2] Bredig: *Zeitschr. f. physikal. chem.*, xiii, p. 191, 1894.

[3] T. B. Osborne: *Journ. Amer. Chem. Soc.*, xxi, p. 486, 1899; *Zeitschr. f. physiol. Chem.*, xxxiii, p. 240, 1901.

[4] W. A. Osborne: *Journ. of Physiol.*, xxvii, p. 398, 1901.

[5] T. Brailsford Robertson: *Journ. of Physical. Chem.*, xiv, p. 528, 1910.

(3). Each equivalent of a mono-acid base neutralized by serum globulin or casein yields *two equivalents* of the protein compound, and, in the presence of excess of acid, each equivalent of a monobasic acid neutralized by ovomucoid also yields two equivalents of the protein compound.[1] This obviously corresponds with the mode of dissociation depicted above, while if terminal $-NH_2$ or $-COOH$ groups accomplished the union each equivalent of neutralised acid or base would produce only *one* equivalent of salt.

(4). On successive addition of 1, 2, 3, etc. equivalents of monoacid bases to a solution of an organic polybasic acid of the type $R\ (COOH)_n$ and the formation of salts by the replacement of H atoms in the $-COOH$ groups, the osmotic pressure of the solution would increase (provided the salts were highly dissociated) in the arithmetical proportion 2:3:4 etc. The experimental fact, for casein, is that the osmotic pressure (= depression of the freezing point) increases in *geometrical* proportion, *i.e.*, each successive equivalent of neutralised base or acid gives rise to the same number of ions. This obviously corresponds with what would be expected were the union and its mode of dissociation of the type outlined above.

(5). It has been pointed out by Vernon[2] that although the power of the sum of the decomposition-products of a protein to neutralize bases is somewhat, yet it is only very slightly greater than that of the unhydrolyzed protein. Now in the process of hydrolysis the -COH.N- groups of the protein are split into $-NH_2$ and -COOH groups; yet this results in no pronounced gain of combining-capacity for bases. The obvious conclusion is that the -COH.N- groups within the protein molecule

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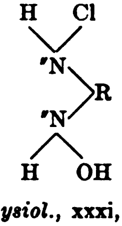
[1] Combined with less acid *four* equivalents of protein salt result from the neutralization of one equivalent of acid. This is due to the formation of ions of the type:—

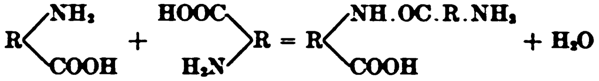


[2] H. M. Vernon: *Journ. of Physiol.*, xxxi, p. 346, 1904.

must be nearly as efficient in accomplishing the neutralization of bases as the $-COOH$ groups of the constituent amino-acids out of which the protein is built up.

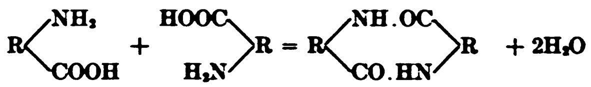
In addition to these, a host of minor details in the behavior of the protein salts, which would be very hard to explain upon any other basis, admit of a simple explanation on the basis of the hypothesis outlined above.

The poly-amino acid structure of the proteins, however, carries with it other possibilities which are of importance in interpreting their behaviour towards precipitating and coagulating agents. The terminal $-NH_2$ and $-COOH$ groups (and at least one of these must exist at either end of a chain of amino-acids) may neutralise themselves internally, thus: $\underbrace{H_2N - R - COO}$ forming what Winkelblech terms an "internal salt."[1] Such a molecule, whatever the *mode* of union, is of course, not able to form *dissociated* salts with acids or bases until the ring formation is opened up. The internal salt formation may go a step further with the formation of *anhydrides* such as $R\begin{matrix}\diagup NH \\ | \\ \diagdown CO\end{matrix}$, such anhydride formation being frequently observed in the polypeptids. Then two molecules of an amino-acid and, therefore, of a polyamino-acid such as protein, may unite with one another in either of two ways, thus:



$$R\begin{matrix}\diagup NH_2 \\ \diagdown COOH\end{matrix} + \begin{matrix}HOOC\diagdown \\ H_2N\diagup\end{matrix}R = R\begin{matrix}\diagup NH.OC.R.NH_2 \\ \diagdown COOH\end{matrix} + H_2O$$

or—



$$R\begin{matrix}\diagup NH_2 \\ \diagdown COOH\end{matrix} + \begin{matrix}HOOC\diagdown \\ H_2N\diagup\end{matrix}R = R\begin{matrix}\diagup NH.OC\diagdown \\ \diagdown CO.HN\diagup\end{matrix}R + 2H_2O$$

the product being, in the first instance, a polyamino-acid of a higher order and greater molecular weight, and in the second an — "internal salt" or anhydride.[2]

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[1] Winkelblech: *Zeitschr. f. physik. Chem.*, xxxiv, p. 564, 1901.

[2] Analogous to leucyl-glycin anhydride.

THE CHEMICAL MECHANICS OF THE PRECIPITATION AND COAGULATION OF PROTEINS BY SALTS

We have seen that in order that *precipitation* of a protein by salts may occur the protein must be ionised, but for *coagulation* this condition is not requisite. In determining the rate of precipitation the valency of the precipitating ion is of prime importance, in determining the rate of coagulation it is of comparatively subordinate importance. For precipitation very low concentrations of the precipitating salt suffice, for coagulation high concentrations of the salt are required. This latter fact, and the fact that the presence of coagulating salts aids coagulation by alcohol and by heat suggests, as it did to Hofmeister, that coagulation is dependent upon *dehydration* of the protein.

Starting from the observation of Jones and Ota[1] that certain salts, when dissolved in water, produce an abnormal depression of the freezing-point, Jones and his pupils have built up a very large body of evidence for the existence of hydrates (or "solvates") of substances in solution; of this evidence a general review will be found in a recent paper by Jones.[2] These investigators find that both ions and undissociated molecules can form "solvates" and that these hydrates or "solvates" are readily decomposed at temperatures which approach the boiling-point of the solvent and by the presence of other agents in the solution which compete for the solvent.[3] An interesting attempt has been made, upon the basis of this hypothesis, to explain the color changes which many salts undergo in the presence of varying amounts of water or of dehydrating agents[1]. As is well known, anhydrous cobalt chloride is blue, but on taking up water it becomes violet or red. Ostwald[2] believed that the undissociated cobalt chloride is blue, while the cobalt ion is red. Since, however, the color of a concentrated solution of cobalt chloride can be changed from purplish-red to blue by the addition of small amounts of calcium, or still smaller amounts of aluminium chlorides, or by the addition of a few drops of alcohol[3] Lewis concludes that this change in color is due to dehydration of the cobalt chloride molecule in solution, by the abstraction of water from it by the added substance. Similar conclusions had previously been reached by other observers.[4]

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[1] H. C. Jones and Ota: *Amer. Chem. Journ.*, xxii, 1899.

[2] H. C. Jones: *Zeitschr. f. physik. Chem.*, lxxiv, p. 325, 1910, cf. also Walden: *Trans. Faraday Soc.*, vi, p. 71, 1910.

[3] A very striking experiment illustrating the formation of "solvates" is that cited by Pickering (*Ber. d. d. chem. Ges.*, xxiv, p. 3639, 1891). If a mixture of propyl alcohol and water be placed in a semipermeable vessel and surrounded with water, it is found that water enters the cell, but that no propyl alcohol escapes. If, however, the same semipermeable vessel, containing the same mixture of alcohol and water be immersed in propyl alcohol, propyl alcohol enters the cell and water does not leave it. In other words the vessel is permeable to either propyl alcohol or water when these are pure, but it is impermeable to a mixture of the two, the inference being that large molecular complexes are formed on mixing these reagents, which cannot pass through the pores of the vessel. From these and similar experiments Poynting (*Phil. Mag.*, xlii, p. 298, 1896) concludes

Similarly the progressive change in color of cupric chloride solutions, from blue to greenish brown, on concentration or dehydration is attributed to the loss of water on the part of cupric chloride-water complexes. Lewis finds that if various bromides be added to concentrated solutions of cupric bromide the copper salt is dehydrated (turned brown) by the salts of monovalent metals in the order $Li > (Na, NH_4) > K$. For the chlorides the order was $Li > Na > NH_4 > K$. Divalent metals dehydrate more strongly, the order being $Mg > Ca > Gr > Ba$ while trivalent metals (*Al*) act still more energetically. In opposition to this view Donnan[5]

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that osmotic pressure is an expression of the diminution in the active mass of the solvent due to the formation of compounds with the dissolved substance.

[1] H. C. Jones and Uhler: *Amer. Chem. Journ.*, xxxix, p. 291, 1905; xxxvii, p. 126, 1907; H. C. Jones and Anderson: *Ibid.*, xli, p. 163, 1909; H. C. Jones and Strong: *Ibid.*, xliii, pp. 37 and 224, 1910; G. N. Lewis: *Zeitschr. f. physik. Chem.*, lii, p. 224, 1905; lvi, p. 223, 1906; H. C. Jones and Anderson: *Carnegie Inst. Publ.* 110.

[2] Wilh. Ostwald: *Grundlinien d. anorg. Chem.*, p. 620.

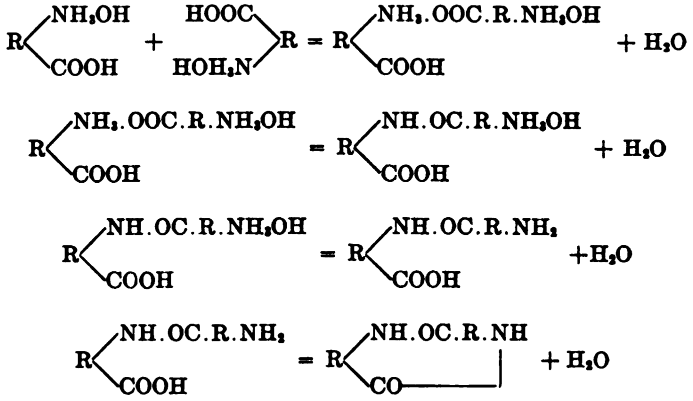
[3] Babo: Cf. *Jahresber. d. Chem.*, 1857, p. 72.

[4] Russell: *Proc. Roy. Soc. Lond.*, xxxii, p. 258, 1881; Potilitzin: *Ber. d. d. chem. Ges.*, xvii, p. 276, 1884; *Bull. soc. chim.*, (3) vi, p. 264, 1891; Wyrouboff: *Bull. soc. chim.* (3) v, p. 460, 1891; p. 3, vi, 1891; LeChatelier: *Bull. soc. chim.* (3) vi, p. 84, 1891; Hartley: *Trans. Roy. Soc. Dublin*, ii, 7 p. 253, 1900; *Journ. Chem. Soc.*, 83, p. 401, 1903.

[5] Donnan and Basset: *Journ. Chem. Soc.*, lxxxi, p. 942, 1902; Donnan: *Zeitschr. f. physik. Chem.*, liii, p. 317, 1905; Cf. also M. Lewin: *Ibid.*, iv, p. 513, 1906; Moore: *Ibid.*, lv, p. 641, 1906; Denham: *Ibid.*, lxv, p. 641, 1909.

advances the hypothesis that the blue color of concentrated solutions of cobalt chloride is due to the formation of complex anions of the type $CoCl_2.Cl_2''$. Lewis points out, however, that this view is inconsistent with the fact that it is possible to change the color of the solution from blue to red by mere dilution, without altering the active mass of any component of the system except water. Other objections against Donnan's view have been urged by Hartley and by H. C. Jones.

The peculiar interest to the biological chemist of the possibility thus indicated, that substances dissolved in water form loose combinations with the solvent, lies in the especial significance of water in relation to the protein and polypeptid structure. As indicated above, dehydration of a protein may result in the following reactions:—


R<NH$_3$OH / COOH + HOOC / HOH$_3$N>R = R<NH$_3$.OOC.R.NH$_3$OH / COOH + H$_2$O
R<NH$_3$.OOC.R.NH$_3$OH / COOH = R<NH.OC.R.NH$_3$OH / COOH + H$_2$O
R<NH.OC.R.NH$_3$OH / COOH = R<NH.OC.R.NH$_2$ / COOH +H$_2$O
R<NH.OC.R.NH$_2$ / COOH = R<NH.OC.R.NH / CO + H$_2$O

and hydration, of course, may result in the reversion of this series of changes.

That proteins may be thrown out of solution in the very different conditions of hydration is evident from the researches cited in the earlier part of the paper; it is even more clearly shown by the following experiments:—

Anhydrous casein dissolves readily in cold anhydrous[1] formic acid; still more readily in hot formic acid. If, to a 2 per cent solution of casein in formic acid, we add a fairly concentrated solution of cupric chloride the mixture is at first green, indicating the presence of lower hydrates of cupric chloride, but on adding more of the solution it becomes blue, *and simultaneously with the appearance of a pure blue color, but not before, precipitation of cupric caseinate occurs.* If to 5 cc. of a 2 per cent solution of casein in formic acid, we add 1.5, 2.0 or 2.5 cc. of a saturated solution of cupric chloride, no precipitation of the caseinate occurs but on diluting this mixture with water a precipitate results, and the appearance of this precipitate coincides with the attainment of a clear blue color on the part of the mixture.

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[1] Anhydrous, that is, save for traces of moisture derived from the atmosphere.

About 6 cc. of water are required to produce a permanent precipitate. This precipitate redissolves on heating and *the mixture simultaneously becomes green;* on cooling the blue color reappears and with it the precipitate. If formic acid be added to the mixture the precipitate redissolves as soon as the mixture becomes green. If the precipitate be very slight it will redissolve on adding alcohol. It cannot be urged that the formation of the cupric caseinate requires the presence of a sufficient concentration of cupric ions, because green solutions of cupric chloride contain an abundance of ions,[1] and casein will react with very small amounts of metal ions, for although it is itself insoluble, it will drive carbonic acid out of the sparingly soluble calcium carbonate to form a freely soluble caseinate of calcium.

If, instead of adding *water* to a mixture of 5 cc. of 2 per cent casein in formic acid and 2 cc. of saturated $CuCl_2$, we add *alcohol,* no coagulation occurs until the mixture changes in color from green to brown when a coagulum of cupric caseinate is produced which redissolves on adding water.

Similar results are obtained when a 2M solution of cobalt chloride is employed instead of a saturated solution of cupric chloride. If to 5 cc. of a 2 per cent solution of casein in formic acid we add

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[1] Green solutions containing, probably, a mixture of the anhydrous brown salt and the fully hydrated blue salt. Even on the basis of the hypothesis urged by Donnan (Cf. above), therefore, a considerable number of cupric ions must exist in green solutions. It is important to notice that precipitation, as stated above, does not occur until the solution is *pure* blue in color; mixtures so slightly green that they appear wholly blue until viewed alongside a pure blue mixture produce no precipitate.

2 to 3 cc. of 2M $CoCl_2$ we obtain a blue-purple solution; on adding water to this mixture it changes in color from blue-purple through red-purple to clear pink. Not until a pure pink color is obtained does a precipitate result. If, instead of adding water we add a very considerable volume of alcohol (10 volumes) the mixture rather abruptly changes to a clear pale blue and then, but not before, we obtain a *coagulum* of cobalt caseinate.

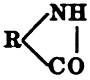
Electro-negative casein is not precipitated by the salts of the alkalies, though it is readily precipitated by salts of the alkaline earths. Electro-positive casein (*i.e.*, casein dissolved in acids) is, however, very readily precipitated by salts and these precipitates are not soluble upon dilution. Thus if 2 cc. of $\frac{N}{10}$ HCl be added to 5 cc. of a 1 per cent solution of casein in 0.008 N KOH, a clear acid solution of casein results. The casein is precipitated from this by the addition of four drops of a saturated solution of sodium chloride, or by one drop of a saturated solution of ammonium sulphate; this latter precipitate does not dissolve on diluting the mixture sixteen times.

Casein formate is no exception to the other salts which casein forms with acids, but *the precipitation will only occur in the presence of a sufficiency of water.* If to 5 cc. of a 2 per cent solution of casein we add a saturated solution of ammonium sulphate, 3 cc. of this solution just suffice to produce a coagulum, this becomes more abundant on adding water, and redissolves on adding formic acid. If, however, instead of 3 cc., we add 2 cc. of the saturated ammonium sulphate solution, a clear solution is obtained. *On adding water to this a precipitate results which redissolves on heating and reappears on cooling.* If, instead of adding water we add alcohol, 5 cc. suffice to produce a voluminous coagulum which redissolves on adding 5 cc. of water; but on adding 10 additional cc. of water the protein is thrown out of solution again.

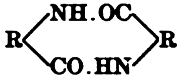
Analogous results may be obtained with ovomucoid.

It is clear, therefore, that protein may be thrown out of solution by electrolytes in two grades of hydration, the one of high, and the other of very low hydration. The former process is what we have termed precipitation, the latter we have defined as coagulation. At grades of hydration intermediate between the extremes the protein may be soluble. Dehydration, partial or complete, leading to resolution or coagulation may be induced by heat, by non-electrolytes possessing an affinity for water or by electrolytes.

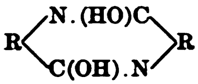
The importance of a high degree of dehydration in the production of *coagula* irresistibly suggests that this phenomenon is dependent upon the formation of anhydrides[1] analogous to leucyl-glycin anhydride and of the general formula:



or

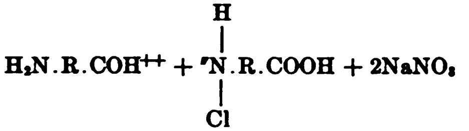


Such bodies may exist either in the -keto form, illustrated by the above formula, or in the -enol form, such as:

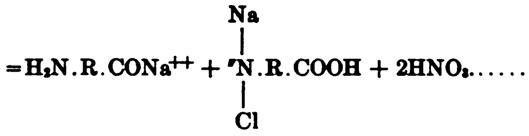


If this be granted then the fact that alcohol throws down the protein salts in an unaltered condition[2] lends strong support to the views which I have advanced regarding the mode of formation and structure of the protein salts, since according to that view the metal ions in a protein-metal compound are, previously to dehydration bound up in -enol groups, and dehydration, which would only affect terminal $-NH_2$ or $-NH_3OH$ and $-COOH$ groups would leave the union between the protein and the metal unaffected.

As regards the *precipitation* of proteins by salts it appears probable, that acid and alkali protein react with salts as follows:—



$$H_2N.R.COH^{++} + \overset{H}{\underset{Cl}{{}'N}}.R.COOH + 2NaNO_3$$



$$= H_2N.R.CONa^{++} + \overset{Na}{\underset{Cl}{{}'N}}.R.COOH + 2HNO_3 \ldots\ldots\ldots (1)$$

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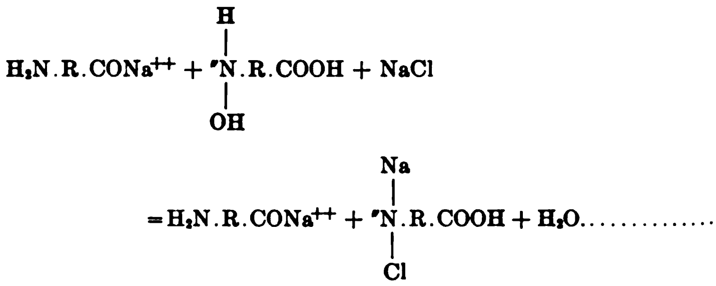
[1] Cf. also Gustav Mann: *Physiological Histology*, Oxford, 1902.

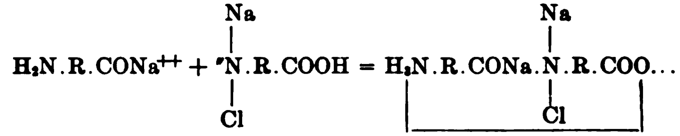
[2] T. Brailsford Robertson: *Journ. of Physical Chem.*, xv, 1911 "Theories in the Electrochemistry of the Proteins." Part vi.



$$\mathrm{H_2N.R.CONa^{++} + {}'\overset{H}{\underset{OH}{N}}.R.COOH + NaCl}$$

$$\mathrm{= H_2N.R.CONa^{++} + {}'\overset{Na}{\underset{Cl}{N}}.R.COOH + H_2O} \ldots\ldots\ldots\ldots\ldots (2)$$

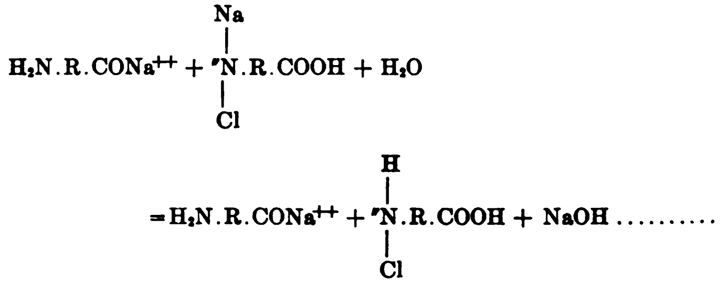
Since proteins, dissolved in salt solutions, are electrically neutral (Hardy) it appears probable that this compound undergoes internal neutralisation thus:—



$$\mathrm{H_2N.R.CONa^{++} + {}'\overset{Na}{\underset{Cl}{N}}.R.COOH = H_2N.R.CONa.\overset{Na}{\underset{Cl}{N}}.R.COO} \ldots (3)$$

It will be observed that this hypothesis is a slight modification of that advanced by Pauli and Handovsky (cf. above) in that, in the first place, cognisance is taken of the fact that the proteins ionize, not at terminal NH$_2$ or -COOH groups but at internal -enol groups and, in the second place, the compounds which are formed with acid and alkali protein respectively are symmetrical in structure, so that the symmetry of the effects of salts in dissolving and coagulating these compounds, which has been observed by Pauli, is readily accounted for.

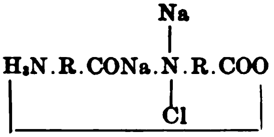
In dilute solutions, that is, solutions in which the *active mass of water* is great, these compounds undergo hydrolytic dissociation in the following way:—



$$\mathrm{H_2N.R.CONa^{++} + {}'\overset{Na}{\underset{Cl}{N}}.R.COOH + H_2O}$$

$$\mathrm{= H_2N.R.CONa^{++} + {}'\overset{H}{\underset{Cl}{N}}.R.COOH + NaOH} \ldots\ldots\ldots\ldots (4)$$

This decomposition will naturally take place more readily in cid than in alkaline solution; the more concentrated acid would of ourse abstract Na from the compound and convert the whole of into the acid-protein compound. In either case the neutral ompound which results may be insoluble. When the active mass f water is diminished, however, for example by the addition of lehydrating agent or a salt with an affinity for water, this hydrolytic lecomposition is prevented, and the complex salt:



$$H_2N.R.CONa.\overset{Na}{\underset{Cl}{N}}.R.COO$$

ay pass into solution.

Further dehydration leads to the loss of -H and -OH by terminal NH_2 and -COOH groups, as depicted above, and the formation of omplex insoluble anhydrides.

This hypothesis furnishes an explanation of the following facts.

(1). That the addition of salts to a solution of acid-protein creases the acidity of the solution, while the addition of salts to kali-protein solutions does not increase the alkalinity (vide uations 1 and 2).

(2). That an acid-protein is precipitated by cations, alkali-rotein by anions (vide equation 4).

(3). The reaction (acidity or alkalinity) of the system being ept constant, the precipitation of the protein depends only upon e active mass of water and not upon the active mass of salt, rovided this is sufficient to enter into combination with the pro-in (vide equation 4), *i.e.*, it is possible to bring about precipitation y mere dilution, the relative masses of protein and salt being naltered.

(4). The precipitation of proteins by salts occurs more readily acid than in alkaline solutions (vide equation 4).

(5). The observation of Bonamartini and Lombardi[1] that egg lbumin in neutral solution combines with both the basic and acid radicals of copper sulphate in equivalent proportions to form an insoluble compound but that in alkaline solution it combines with excess of copper to form a soluble compound (vide equation 4). In alkaline solution hydrolytic dissociation of the complex salt is pushed back in accordance with the following equations:

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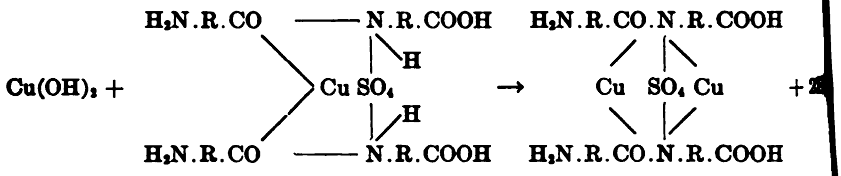
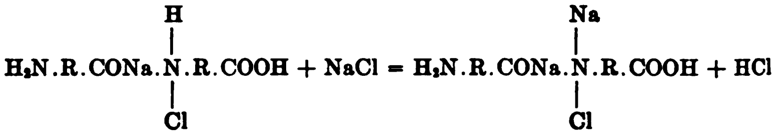
[1] Bonamartin and Lombardi: *Zeitschr. f. physiol. Chem.*, lviii, p. 165, 08. The egg albumin employed by these observers is not ash-free; it ust therefore according to Pauli have been ionic.

$$CuSO_4 + 2KOH \rightarrow K_2SO_4 + Cu\,(OH)_2$$



(6). The observation of T. B. Osborne that edestin, crystallised from concentrated salt-solutions, will decompose the salt, binding the base, is probably attributable to the reaction:



$$H_2N.R.CONa.\overset{\overset{H}{|}}{\underset{\underset{Cl}{|}}{N}}.R.COOH + NaCl = H_2N.R.CONa.\overset{\overset{Na}{|}}{\underset{\underset{Cl}{|}}{N}}.R.COOH + HCl$$

the complex salt being in this case, insoluble.

(7). The observation of Pauli (*loc. cit.*) that *precipitation* of a protein by salts when it is non-ionic is impossible. For, when the protein is not ionised the nitrogen is bound up in undissociated -COH.N- groups and is not attached to H and OH groups replacable by the ions of the salt.

This fact also explains the observation of Liebermann and Bugarsky[1] that uncombined and, presumably, unionised egg-albumin does not combine with salts in neutral solutions.

(8). The observation of Hardy (*loc. cit.*) and Pauli (*loc. cit.*) that coagulation of a protein by salts is possible whether it is ionic or not, since the dehydration of terminal -NH_2 and -COOH groups does not depend upon the dissociation of -COH.N- groups.

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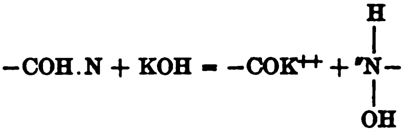
[1] Bugarszky and Liebermann: *Arch. f. d. ges. Physiol.*, lxxii, p. 51, 1898. These observers used egg-albumin which, according to Pauli, is electrically neutral when uncombined with acids or bases.

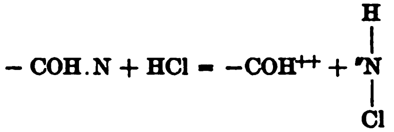
SUMMARY.

Evidence is advanced in support of the following theses:—

1. The proteins do not combine with acids and bases through the agency of terminal -NH_2 and -COOH groups, but through the agency of -COH.N- groups, according to equations of the types:—

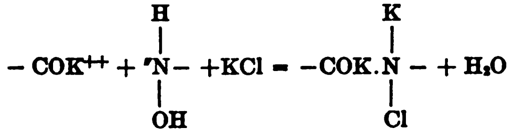


$$-\mathrm{COH.N} + \mathrm{KOH} = -\mathrm{COK}^{++} + {}^{\prime}\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{N}}}-$$

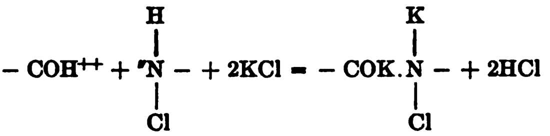


$$-\mathrm{COH.N} + \mathrm{HCl} = -\mathrm{COH}^{++} + {}^{\prime}\underset{\mathrm{Cl}}{\overset{\mathrm{H}}{\mathrm{N}}}$$

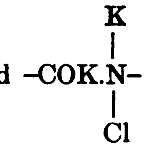
2. These compounds react with salts, in solution, according to the equations:—



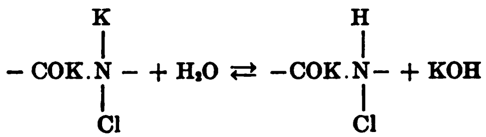
$$-\mathrm{COK}^{++} + {}^{\prime}\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{N}}}- + \mathrm{KCl} = -\mathrm{COK.}\underset{\mathrm{Cl}}{\overset{\mathrm{K}}{\mathrm{N}}} - + H_2O$$



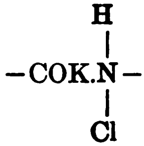
$$-\mathrm{COH}^{++} + {}^{\prime}\underset{\mathrm{Cl}}{\overset{\mathrm{H}}{\mathrm{N}}} - + 2\mathrm{KCl} = -\mathrm{COK.}\underset{\mathrm{Cl}}{\overset{\mathrm{K}}{\mathrm{N}}} - + 2\mathrm{HCl}$$



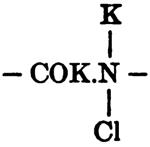
3. The compound $-\mathrm{COK.}\underset{\mathrm{Cl}}{\overset{\mathrm{K}}{\mathrm{N}}}-$ in dilute and especially in acid solution tends to undergo hydrolytic dissociation according to the equation:



$$-\mathrm{COK.}\underset{\mathrm{Cl}}{\overset{\mathrm{K}}{\mathrm{N}}} - + H_2O \rightleftarrows -\mathrm{COK.}\underset{\mathrm{Cl}}{\overset{\mathrm{H}}{\mathrm{N}}}- + \mathrm{KOH}$$

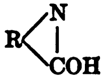


4. The compound $-\mathrm{COK.}\overset{\overset{\mathrm{H}}{|}}{\underset{\underset{\mathrm{Cl}}{|}}{\mathrm{N}}}-$ is usually insoluble; the formation of this compound results in the *precipitation* (as distinguished from *coagulation*) of the protein.



5. In the presence of a greater mass of the salt or of a moderate amount of other dehydrating agents the above balanced reaction is reversed and we regain the compound: $-\mathrm{COK.}\overset{\overset{\mathrm{K}}{|}}{\underset{\underset{\mathrm{Cl}}{|}}{\mathrm{N}}}-$ which is usually soluble.

6. In the presence of still greater quantities of dehydrating agents or on heating, the terminal -NH_2 and -COOH groups of the protein molecule react with one another, yielding water and anhydrides of the types:



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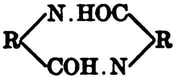

which, whether or not combined with bases or acids, are usually insoluble. This results in the *coagulation* (as distinguished from the *precipitation*) of the protein.

NOTE UPON RELATIONSHIP BETWEEN UREA AND AMMONIUM SALTS.

By A. J. WAKEMAN and H. D. DAKIN.

(*From the Herter Laboratory, 819 Madison Avenue, New York.*)

(Received for publication, April 12, 1911.)

An ever increasing number of the simpler chemical reactions occurring in the animal body is being shown to be of a reversible type. It appeared to the writers to be of interest to determine whether under suitable conditions the reaction involved in the well known conversion of ammonium carbonate or carbamate into urea by the liver might not also be a reversible reaction. An additional incentive for making these experiments was afforded by the suggestion of some writers[1] that some of the conditions of acidosis in which an increased quantity of ammonium salts is excreted in the urine may fundamentally be caused by an accumulation of alkaline ammonium salts which the organism must endeavor to neutralize with acids, rather than by an accumulation of acid which must be neutralized by ammonia.

It is obvious that a demonstration of the reversible character of the reaction involved in the conversion of ammonium carbonate into urea would be of some importance with regard to the study of acidosis. We have made many attempts to demonstrate such a reconversion of urea into ammonium salts but without success.

Our experiments were made by perfusing the surviving livers of dogs with a mixture of dogs' blood and saline to which urea had been added. The concentration of urea varied in the different experiments and in some was raised as high as 4 grams per liter. In some experiments repeated small additions of hydrochloric acid were made to the perfusion fluid after each passage through the liver. It was thought that these conditions might possibly favor ammonia production. Estimations of the amount of ammonia in the blood were made immediately prior to the commencement of the perfusion and also after perfusion had been carried on for one hour. In every case there was a complete failure to demonstrate any increased formation of ammonia at the expense of the urea. Under the conditions of our experiments the conversion of ammonium carbonate into urea appears to be practically an irreversible reaction.

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[1] Cf. Ewing: *Amer. Journ. of Med. Sciences*, June, 1910.

FORMIC ACID AS AN INTERMEDIARY SUBSTANCE IN THE CATABOLISM OF FATTY ACIDS AND OTHER SUBSTANCES.

PRELIMINARY NOTE.

By H. D. DAKIN AND A. J. WAKEMAN.

(From the Herter Laboratory, 819 Madison Avenue, New York.)

(Received for publication, April 12, 1911.)

A small amount of formic acid has long been recognized as a normal urinary constituent, but the origin of the acid has not been satisfactorily determined. Since formic acid is oxidized moderately easily in the animal body it is fair to conclude that the actual amount of formic acid produced in the course of metabolism is considerably in excess of the quantity excreted in the urine. It is therefore a matter of some interest to endeavor to determine the conditions of its formation.

The writers are engaged in perfecting a method for the determination of formic acid in urine, and similar fluids, which they believe to possess advantages over the customary procedures. With the aid of this method we have carried out a number of determinations of formic acid in normal and pathological urines. We have also found that a relatively large excretion of formic acid follows the intravenous administration to cats of the sodium salts of fatty acids such as acetic, propionic, butyric, caproic etc. The urines excreted after the administration of these salts naturally contain much sodium bicarbonate and for purposes of control we have injected normal cats with solutions of equivalent quantities of sodium bicarbonate. *Under these cricumstances we find that the administration of the sodium salts of the fatty acids mentioned may result in the excretion of from ten to thirty times the normal amount of formic acid.*

From these results it appears probable that formic acid may represent a stage in the catabolism of the fatty acids from acetic acid upwards. The writers are engaged upon experiments aiming at the solution of this question. The relation of carbohydrates and amino-acids to formic acid production is also under investigation. The experimental results will be published shortly.

SULPHUR IN PROTEINS.

THIOPOLYPEPTIDES.

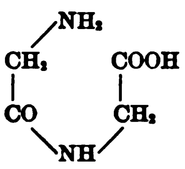
Preliminary Paper.

By TREAT B. JOHNSON and GERALD BURNHAM.

(*Contributions from the Sheffield Laboratory of Yale University.*)

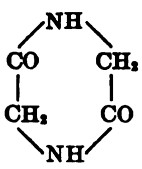
(Received for publication, April 12, 1911.)

It is generally admitted, at the present time, that the amino-acids, obtained as cleavage products in protein hydrolysis, exist in the original proteins as imino compounds, viz.: polypeptides (I) or their anhydrides (II).



I

Glycylglycin



II

Dioxypiperasin

It is also a well known fact that all proteins which have been carefully examined, with the exception of the protamines and certain bacterial proteins, contain sulphur. This element occurs in varying amounts in different proteins, but attains apparently a maximum percentage in the horn substances.

We have, however, at the present time, very little knowledge of the true nature of the sulphur linkings in these natural substances. Admitting that cystine has been isolated from several sulphur proteins and is looked upon as a primary dissociation product, nevertheless there are good reasons for believing that this is not the only primary sulphur complex to be considered. Considering only the oxygen linkings of the various acid products of protein hydrolysis, there are just two oxygen groupings to be dealt with, viz.: the hydroxyl form OH as represented in serine, oxyproline and tyrosine (IV), and the keto form –CO–NH and COOH– which is present in the polypeptides (I), or their anhydrides (II) and the acids themselves. If now sulphur proteins be

$$\underset{\text{III}}{\mathrm{HO.CH_2CH(NH_2)COOH}} \qquad \underset{\text{IV}}{\mathrm{HOC_6H_4CH_2CH(NH_2)COOH}}$$

$$\underset{\text{V}}{\mathrm{HSCH_2CH(NH_2)COOH}}$$

viewed as oxygen proteins in which bivalent sulphur has partially displaced oxygen, one might therefore expect to find two types of sulphur linkings in sulphur proteins corresponding to the two important, primary oxygen groupings, viz.: mercapto –SH and thioamide –CS–NH–. In fact cystein (V) represents a sulphur compound of the first type, in which such a displacement has taken place in the case of hydroxyl oxygen in serine (III). Thiotyrosine, HS.$C_6H_4CH_2CH(NH_2)COOH$, has not yet been synthesized.

Are *thiopolypeptide* linkings present in sulphur proteins? If not, do thioamides functionate at all in the natural synthesis of sulphur proteins from simpler substances? Judging from some results which have already been obtained in this laboratory, it seems not improbable to the writer that *thiopolypeptide* groupings –NH.CH_2.CS– may be present in some sulphur proteins, and that a knowledge of this grouping is therefore very desirable in order to help to explain many of the interesting changes of these natural substances.

We attach much importance to this new idea of thiopolypeptide sulphur. Thiopolypeptides represent a new class of organic compounds and we have already prepared several interesting derivatives of them, and are making a careful study of their chemical properties. This work is now in progress in this laboratory.

HYDROLYSIS OF CASEIN.[1]

By THOMAS B. OSBORNE and H. H. GUEST.

(From the Laboratory of the Connecticut Agricultural Experiment Station.)

(Received for publication, April 13, 1911.)

During the last few years the proportion of amino-acids yielded by the more important proteins has been estimated by the methods now available. While these analyses have indicated important differences in the proportion of some of the amino-acids yielded by different proteins, nevertheless, from a quantitative standpoint so much uncertainty still attaches to many of the determinations that these analyses have only a limited value when applied to the solution of questions concerning nutrition or physiology.

The fundamental importance of a definite knowledge of the quantity of each amino-acid yielded by the several food proteins justifies the expenditure of much effort in studying the analytical methods in order that these may be improved, or their limitations definitely ascertained. It is evident that a satisfactory quantitative knowledge of the products of protein hydrolysis is to be ultimately obtained only by continued efforts to improve the methods of isolating the various amino-acids until the sum of the quantity of these becomes nearly, if not quite, equal to 100 per cent.

The results of a beginning of a study, directed to this end, reported in earlier papers from this laboratory, were obtained by analyzing the products of hydrolysis of zein from the seeds of maize, a protein which contains no glycocoll, tryptophane, lysine, or carbohydrate and only a small proportion of arginine and histidine. Many ways were found to increase the yields of several of the amino-acids so that the total summation of these, isolated in a pure con-

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[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

dition, was so much increased as to materially add to the value of the analysis. At the same time the probable errors of the analysis were determined and by taking these into consideration it was made highly probable that nearly all of the products of hydrolysis of zein as well as their approximate proportions are now known. With the expectation that we might accomplish at least as much for casein, we undertook the analysis described in this paper.

Although our attempt to effect a satisfactory analysis of casein failed to give the results hoped for, we, nevertheless, give them in this paper since they help to explain some of the conflicting data now recorded and at the same time afford an opportunity to discuss the present state of knowledge of the decomposition products of this important protein. They also give some new information respecting the losses incident to the conduct of the analytical methods and suggest possibilities for improving these.

The only summation of the products of hydrolysis of casein is that published by Abderhalden[1] the total of which is but little more than 50 per cent. A number of isolated data concerning the proportion of individual amino-acids are to be found in the literature but as these have never been brought together they will be later discussed in connection with our own determinations.

PREPARATION OF CASEIN.

The casein used for this analysis was prepared in the laboratory according to Hammarsten's method by diluting 40 liters of perfectly fresh, nearly fat-free, centrifugated milk with 100 liters of water and precipitating the casein with the least possible quantity of abundantly diluted acetic acid. After the precipitate had settled the solution was drawn off and the precipitate suspended in about 50 liters of water. The least possible excess of a very dilute solution of sodium hydroxide was then added, with constant stirring, until the casein was dissolved. After diluting to about 150 liters the casein was reprecipitated with acetic acid and this process repeated five times. The final solution in alkali was obtained within eight hours. After standing over night this was filtered *clear* through a thick bed of filter paper pulp and the casein precipitated from a volume of about 150 liters with the least possible quantity of very dilute hydrochloric acid. After the precipitate had settled, the solution was drawn off and again suspended in about 150 liters of water, in order to remove water-soluble substances. The precipitate was then brought on to a linen cloth and allowed to drain until the greater part of the solution had run off. It was then suspended in dilute alcohol and washed chlorine free by draining on linen cloth. The washed casein was next digested with absolute alcohol and then with ether and air-dried. Thus obtained, it formed a snow-white, dusty powder which yielded a perfectly clear solution when dissolved in alkali and gave only the faintest trace of the Molisch reaction.

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[1] Abderhalden: *Zeitschr. f. physiol. Chem.*, xliv, p. 23, 1905.

HYDROLYSIS OF CASEIN.

The method recently described by VanSlyke[1] for determining the proportion of amino-nitrogen in the mixture of protein decomposition products affords a means for following the progress of protein hydrolysis which we have applied to casein and have found that complete decomposition is not effected until the casein is boiled with strong hydrochloric acid for much more than twenty-four hours. Thus five portions of 2 grams each of air-dry casein were treated with 20 cc. of hydrochloric acid, specific gravity 1.1, and the mixture heated to boiling with return condenser. After eight hours one portion was removed from the bath, made up to 100 cc. and the total nitrogen in each of two portions of 10 cc. found to be 0.2800 and 0.2814 gm. The amino nitrogen in two portions of 15 cc. was 0.1586 and 0.1586 gm., being thus equal to 56.5 per cent of the total nitrogen.

By proceeding in the same way the proportion of amino nitrogen, after boiling for twenty-four hours, was 66.7 per cent of the total, after forty-eight hours 71.7, after seventy-two hours 71.7, and after ninety-six hours 70.8 per cent of the total. In making these determinations closely agreeing duplicate results were obtained in each case.

The total nitrogen of casein is 15.6 per cent, 71.7 per cent of which is equal to 11.19 per cent. If to this is added the nitrogen in other forms, as calculated from those determinations which appear to be most nearly correct we find that almost all of the nitrogen of the casein is accounted for as is shown in the following table.

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[1] VanSlyke: *Berichte d. deutsch. Chem. Gesellsch.*, 1910, xliii, p. 3170; this *Journal*, ix, p. 184, 1911.

Partition of nitrogen in Casein.

	PER CENT
Ammonia nitrogen	1.61
⅔ Histidine nitrogen	0.46
¾ Arginine nitrogen	0.92
½ Tryptophane nitrogen	0.10
Proline nitrogen	0.82
Oxyproline nitrogen	0.03
Amino nitrogen	11.19
	15.13

This table indicates that we now know with approximate accuracy the several forms of combination in which the nitrogen occurs in casein. Much of the small deficit is probably to be assigned to tryptophane and oxyproline since no methods are known by which these substances can be quantitatively estimated.

We, therefore, digested 350 gm. of ash- and moisture-free casein with 700 cc. of concentrated hydrochloric acid for five successive days, during which time the mixture was heated to boiling for about seven hours daily, or thirty-seven hours in all.

DETERMINATION OF GLUTAMINIC ACID.

The hydrolysis solution was next made up to one liter and divided into four equal parts. Each of these was concentrated to about one-half its volume, saturated with gaseous hydrochloric acid and placed on ice for twenty-four hours. The glutaminic acid hydrochloride was recrystallized with as little loss as possible. From each of the four portions we thus obtained, 17.2, 16.49, 16.57, 16.47 gm. of glutaminic acid hydrochloride respectively, making a total of 66.73 gm., equal to 53.47 gm. of free glutaminic acid, or 15.28 per cent of the casein. No more was obtained from the esters which were subsequently distilled, indicating a practically complete separation as hydrochloride. An examination of the total quantity of this glutaminic acid hydrochloride in the condition in which it was weighed showed it to be free from moisture and to contain only 0.17 gm. of ammonium chloride. It melted with decomposition at 198°.

Chlorine, 0.2718 gm. subst. gave 0.2173 gm. AgCl. Deducting the small amount of chlorine contained in the ammonium chloride the remainder is equal to 19.61 per cent chlorine.

Calculated for $C_5H_{11}O_4NCl$: Cl = 19.34 per cent
Found: Cl = 19.61 per cent

After hydrolyzing for thirty-two hours 400 gm., ash- and moisture-free, of this same preparation of casein we obtained 74.5 gm. of glutaminic acid hydrochloride and, in addition to this, 3.12 gm. from the esters, making a total of 77.62 gm. equivalent to 62.2 gm. of free glutaminic acid, or 15.55 per cent of the casein. A careful examination of this glutaminic acid hydrochloride showed that it contained no moisture or ammonium chloride.

Analysis of the substance as weighed gave the following results.

Nitrogen, 0.2972 gm. subst. required 16.0 cc. $\frac{N}{10}$HCl.
Calculated for $C_5H_{10}O_4NCl$:......................N = 7.63 per cent
Found:......................................N = 7.54 per cent
Chlorine, 0.2717 gm. subst. gave 0.2142 gm. AgCl = 0.0531 gm. Cl.
Calculated for $C_5H_{10}O_4NCl$:..................Cl = 19.36 per cent
Found :......................................Cl = 19.49 per cent

It melted with decomposition at 199°.

After separating the glutaminic acid hydrochloride, the remaining amino-acids were esterified by the method of Phelps and Phelps and the esters set free with sodium ethylate. After filtering out the sodium chloride and other substances (A) insoluble in alcohol, the alcohol was distilled off under diminished pressure, the distillate acidified with hydrochloric acid, evaporated to dryness, the chlorine removed and the amino-acids added to those from Fractions I and II, obtained by distilling the residual esters.

These esters were treated with dry ether and the soluble part (B) filtered from the insoluble part (C).

The ether was distilled from the esters (B) at atmospheric pressure, the distillate acidified with hydrochloric acid, and the ether distilled off. After removing chlorine from the residue of amino-acids these were added to those from Fractions I and II subsequently obtained by distilling the main portion of the esters (B).

Distillation I. Esters B.

FRACTION	TEMPERATURE OF BATH UP TO	PRESSURE	WEIGHT
		mm.	*gm.*
I	65°	3	27.24
In liquid air condenser			9.00
II	80°	1	52.22
III	105°	1	25.79
IV	160°	1	12.74
In liquid air condenser			16.00
Undistilled residue			70.00
Loss			9 00
			224.00

Fractions I and II together with the 9 gm. from the liquid air condenser yielded 31.35 gm. of amino-acids, insoluble in absolute alcohol. The alcoholic extract was united with the other extracts similarly obtained and worked up for proline as later described.

Fraction III together with the 16.0 gm. from the liquid air condenser yielded 12.55 gm. of amino-acids insoluble in alcohol which, together with those from Fractions I and II, made a total of 43.90 gm. consisting essentially of leucine, valine, and alanine.

Fraction IV. From the ether extract 5.97 gm. of pure phenylalanine hydrochloride were obtained.

From the aqueous solution no glutaminic acid was isolated and only 1.0 gm. of copper aspartate.

The esters (C) insoluble in ether, which presumably were chiefly those of the basic amino-acids, formed a plastic mass from which it appeared difficult to remove all of the ether-soluble esters. To secure these C was dissolved in alcohol, again subjected to the esterifying process and the esters liberated with sodium ethylate and distilled as follows:

Distillation II. Esters C.

FRACTION	TEMPERATURE OF BATH UP TO	PRESSURE	WEIGHT
		mm.	*gm.*
I	120°	20	54
Undistilled residue			33

Fraction I, largely alcohol and ether, yielded only 1.70 gm. of amino-acids insoluble in alcohol. These were added to the corresponding substance obtained from Fractions I and II from Distillation I and the alcoholic extract to the alcoholic extract of the same fractions.

The undistilled residue was treated as Fischer directs for the higher boiling fractions of the esters.[1] The recrystallized phenylalanine hydrochloride from the ether solution weighed 0.77 gm. From the aqueous solution 1.4 gm. of aspartic acid was obtained as the copper salt but no glutaminic acid.

The residue A which contained chiefly sodium chloride and those products of hydrolysis, which failed to be esterified by the first treatment, was freed from inorganic salts by treating with alcoholic hydrochloric acid, the amino-acids esterified, the esters liberated with sodium ethylate and distilled.

Distillation III. Esters A.

FRACTION	TEMPERATURE OF BATH UP TO	PRESSURE	WEIGHT
		mm.	gm.
I	120°	23	38
Undistilled residue			27

Fraction I consisted largely of alcohol and ether for it yielded only 3.63 gm. of amino-acids insoluble in alcohol. These were added to the corresponding substance from Fractions I and II, Distillation I. The alcoholic extract was added to the proline solutions from the same fractions.

The undistilled residue was dissolved in water, shaken with ether and otherwise worked up according to the customary method applied to the higher boiling fractions of the distilled esters. The ethereal solution contained no phenylalanine ester.

From the aqueous solution no glutaminic acid could be separated but 2.88 gm. of aspartic acid was obtained as the copper salt.

The undistilled residue from Distillation I, which weighed 70 gm., was hydrolyzed by boiling with concentrated hydrochloric acid for fifteen hours and then esterified and the esters liberated and distilled.

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[1] Cf. Osborne and Jones: *Amer. Journ. of Physiol.*, xxvi, p. 224, 1910.

Distillation IV.

FRACTION	TEMPERATURE OF BATH UP TO	PRESSURE	WEIGHT
		mm.	gm.
I	125°	17	63
Undistilled residue	....		48

Fraction I, chiefly alcohol and ether, when saponified yielded 6.09 gm. of amino-acids insoluble in alcohol. These were added to the corresponding substance from Distillation I and the alcoholic extract to the proline solution from the same fractions.

The undistilled residue was treated with water and shaken with ether. The ethereal solution yielded 0.92 gm. of phenylalanine hydrochloride. The aqueous solution yielded no glutaminic or aspartic acid.

The amounts of the various amino-acids recovered from each of these distillations is given in the following table.

DISTILLATION	I FIRST ESTERIFICATION	II ETHER INSOLUBLE ESTERS, ETC.	III SECOND ESTERIFICATION	IV UNDISTILLED RESIDUE FROM I	TOTAL
Alanine Valine Leucine	43.9 gm. 79.3%	1.70 gm. 3.0%	3.63 gm. 6.9%	6.09 gm. 11.0%	55.32 gm. 100.0%
Phenylalanine	4.9 gm. 78.0%	0.63 gm. 10.0 %	0.0 gm. 0.0 %	0.75 gm. 11.9 %	6.28 gm. 100.0 %
Aspartic acid	0.48 gm. 10.1%	1.4 gm. 29.5%	2.88 gm. 60.50%	0.0 gm. 0.0 %	4.76 gm. 100.0 %

No account was taken of the quantity of proline obtained from the different fractions as accurate estimations could not be made. The above figures are instructive for they not only show that considerable losses occur in the process of carrying out the analysis but they also show where those losses took place and their relative extent. The figures given under columns II, III, and IV show that, with the exception of aspartic acid, the largest loss was caused by decomposition of the esters during the distillation and that this involved the lower boiling esters to practically the same extent as it did the phenylalanine ester. The next greatest loss, shown under column III, was caused by incomplete esterification and the smallest loss, under column II, occurred in liberating the esters with sodium ethylate.[1] The results obtained for aspartic acid show that it was not as readily esterified as the other amino-acids and that its ester was not easily extracted by ether.

In order to estimate the total quantity of each of the amino-acids contained in the lower boiling fractions of the esters the united fractions from each of the distillations were examined as follows:

THE LEUCINE FRACTION.

The amino-acids insoluble in alcohol from fractions I, II, III, and IV, together with those recovered from the alcohol and ether distilled from the esters, weighed 55.32 gm.

By fractional crystallization 50.33 gm. of amino-acids were obtained which apparently consisted of leucine and valine, and 4.99 gm. which seemed to consist of alanine, but from which no product, sufficiently pure for satisfactory identification, could be isolated. This substance which was probably nearly all alanine was equal to 1.4 per cent of the casein.

The 50.33 gm. of the less soluble amino-acids in which the presence of alanine could not be detected contained carbon, 53.08, and hydrogen, 9.63 per cent, corresponding to a mixture of equal parts of leucine and valine. According to the lead method of Levene and VanSlyke[2] this substance yielded 22.27 gm. of leucine.

The solution of the soluble lead salt yielded 25.04 gm. of valine which gave the following results on analysis.

Carbon and hydrogen, 0.2112 gm. subst. gave 0.3972 gm. CO_2 and 0.1787 gm. H_2O.

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[1] Abderhalden (*Zeitschr. f. physiol. Chem.*, lxviii, p. 477, 1910) found in analyzing the products of decomposition of silk fibroin that the greatest loss occurred in liberating the esters with sodium hydroxide and potassium carbonate.

[2] Levene and VanSlyke: This *Journal*, vi, p. 391, 1909.

Calculated for $C_5H_{11}O_2N$:............... $C = 51.28$; $H = 9.40$ per cent
Found:.............................. $C = 51.29$; $H = 9.47$ per cent

The mixture of 50.3 gm. contained therefore leucine and valine each equal to 7.2 per cent of the casein.

The total alanine, valine, and leucine isolated, namely 15.8 per cent, is somewhat less than that of Levene and VanSlyke[1] who found 18.3 per cent of amino-acids insoluble in alcohol. Their acids contained leucine and isoleucine equal to 9.35 per cent of the casein and valine equal to 6.69 per cent or 16.04 per cent together. This leaves 2.26 per cent of substance, belonging wholly to their fraction I, which must have contained all of the alanine together with a little valine, since, from this fraction they separated 0.5 gm. of pure valine, by direct crystallization, but apparently made no attempt to make the separation of alanine and valine more complete.

As stated on p. 344 the alcoholic extract contained amino nitrogen equal to about 11.0 gm. of amino-acids having a mean nitrogen content of 12 per cent thus making the total amino-acids other than proline belonging to this fraction 66.32 gm. The sum of the alanine, valine and leucine is therefore equal to 19 per cent of the casein.

VanSlyke[2] found the same proportion of amino nitrogen in his alcoholic extract of the amino-acids of the leucine fraction which if added to the 18.3 per cent of amino-acids insoluble in alcohol which Levene and VanSlyke obtained from the same esters would make the sum of the alanine, valine, and leucine which they obtained 21.3 per cent of the casein, a result in substantial agreement with that which we obtained.

Abderhalden[3] gives the proportion of alanine as 0.9 per cent, which doubtless was isolated in a state of purity. Our estimate of 1.4 per cent is based on the weight of the substance from which no more valine could be separated but which could not be converted into a pure enough product for satisfactory identification. All these data show that casein yields a relatively small proportion of alanine which estimated by the present methods is equal to about 1.5 per cent.

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[1] Levene and VanSlyke: This *Journal*, vi, p. 419.

[2] VanSlyke: *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3170, 1910.

[3] Abderhalden: *Zeitschr. f. physiol. Chem.*, xliv, p. 23, 1905.

In commenting on the results of Levene and VanSlyke, Abderhalden[1] has recently said "Casein has been repeatedly analyzed by Emil Fischer and later by myself. The separate estimations gave very accordant results. We obtained a leucine + valine fraction of about 11 gm. Levene and VanSlyke found 16 gm. In spite of the greatest care we have never been able to obtain from casein more than 13 gm. of the leucine fraction per 100 gm. pure casein (Hammarsten)." Our own experience shows that Levene and VanSlyke are more nearly correct than Abderhalden assumes, and that their results may be safely accepted as showing that casein yields at the least as much of the leucines and valine as they have stated.

In this connection it is interesting to note that Engeland[2] obtained results by a wholly different method which indicated that the sum of the leucine and valine yielded by casein is equal to 16.5 per cent. Engeland separated a quantity of "crude leucine" by direct crystallization equal to 13 per cent and from the mother liquor obtained leucine as its trimethyl gold salt equal to about 2 per cent of the casein. From the filtrate from this latter he obtained valine as its trimethyl gold salt equal to 1.5 per cent of the casein. On the assumption that the "crude leucine," separated by direct crystallization, contained approximately equal parts of leucine and valine, Engeland's results agree with those of Levene and VanSlyke as well as with ours. In view of these facts it seems justifiable to accept Levene and VanSlyke's figure of 9.35 per cent as the best determination yet obtained for the proportion of the leucines yielded by casein. Our figure of 7.2 per cent for valine seems to us to be more nearly correct than the slightly lower one of Levene and VanSlyke for ours is based on the weight of analysis-pure substance corresponding to this percentage, and, furthermore, Levene and VanSlyke do not appear to have separated quite all of the valine from the alanine.

The most striking feature of these results for valine is the fact that this substance is present in the products of hydrolysis of casein in so much greater proportion than has heretofore been supposed.

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[1] Abderhalden: *Zeitschr. f. physiol. Chem.*, lxviii, p. 477, 1910.

[2] Engeland: *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 2962, 1909.

On the basis of the data at present available, casein now appears as yielding more valine than any protein yet analyzed.. The recorded data for other proteins, however, are not to be accepted as final for there is little doubt that, by the application of the lead method of Levene and VanSlyke, the amount of valine to be obtained from many of these will be much greater than that heretofore isolated by direct crystallization.

PROLINE.

The united alcoholic solutions containing the proline from the different distillations were worked up in the usual way and 22.86 gm. of alcohol-soluble copper salt and 1.69 gm. of alcohol-insoluble copper salt were obtained.

These copper salts were united and examined according to the method of VanSlyke[1] for determining amino-nitrogen.

The total nitrogen was 3.2704 gm., the amino nitrogen 1.2655 gm., making the difference 2.0049 gm. which corresponds to 16.44 gm. of proline or 4.70 per cent of the casein.

Although we removed from the proline all substances insoluble in absolute alcohol by repeatedly evaporating, redissolving the residue in absolute alcohol and letting the solution stand for a long time it nevertheless contained amino nitrogen equal to 38.7 per cent of its total nitrogen, which agrees with the experience of Van Slyke[2] who similarly found 30.6 per cent. Even after estimating the proline from the non-amino nitrogen the quantity, 4.7 per cent is greater than the 3.1 per cent reported by Abderhalden[3] who did not apply any correction for contaminations. VanSlyke obtained 6.7 per cent of proline from casein as estimated from the non-amino nitrogen soluble in alcohol and Engeland[4] likewise obtained 6.7 per cent by means of the N-methylhygrinic acid.

It is to be noted that VanSlyke's result for proline, which was obtained from the same esters as the leucine and valine estimations of Levene and VanSlyke, is higher than our result by about the same relative amount as their result for the leucines is higher than ours, doubtless because their esterification or distillation was somewhat more successful than ours.

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[1] VanSlyke: *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3170, 1910.

[2] VanSlyke: *Ibid.*

[3] Abderhalden: *Zeitschr. f. physiol. Chem.*, xliv, p. 23, 1905.

[4] Engeland: *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 2962, 1909.

PHENYLALANINE.

The total quantity of phenylalanine isolated from the several distillations, as shown in the table on p. 340 is 6.28 gm.

From the united mother liquors from which the above quantity was obtained 2.00 gm. more were separated as the hydrochloride, thus making a total of 8.28 gm., equal to 2.37 per cent. The total hydrochloride from which the above quantity was estimated was analyzed with the following results.

Chlorine, 0.1683 gm. subst. gave 0.1195 gm. AgCl.
Calculated for $C_9H_{12}O_2$ NCl:..................Cl = 17.61 per cent
Found :....................................Cl = 17.56 per cent

The only record of the percentage of phenylalanine yielded by casein which we have found is given by Abderhalden[1] who attributes its origin to Fischer.[2] Fischer, however, gives no data in the paper referred to from which the proportion of phenylalanine can be inferred further than that it cannot amount to less than 2.5 per cent of the casein. In view of the absence of more definite data we cannot do otherwise than accept the figure which Abderhalden gives as more nearly correct than our own. As we weighed only the pure recrystallized hydrochloride of phenylalanine the amount actually yielded by casein must have been at least as great as 3.2 per cent.[3]

GLUTAMINIC ACID.

Many figures are on record showing the proportion of glutaminic acid yielded by casein. The older figures, obtained by methods now known to give unreliable results require no comment, but the more recent data deserve especial consideration since many of them agree with one another so closely that they have been generally accepted as representing very nearly the true amount of glutaminic acid yielded by casein. Thus Emil Fischer[1] obtained 10 per cent; Abderhalden[2] 10.7; Osborne and Gilbert[3] 10.8; while Abderhalden and Funk[4] state that they have never found more than 10–11 per cent and that they obtained the same amount after boiling the casein with concentrated hydrochloric acid or with 25 per cent sulphuric acid for sixteen hours. Engeland[5] obtained from casein by means of the hydrochloride 9.4 per cent of glutaminic acid. Recently Abderhalden and Langstein[6] have confirmed these data and report 10.78 per cent as the result of a new determination.

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[1] Abderhalden: *Zeitschr. f. physiol. Chem.*, xliv, p. 23, 1905.

[2] Fischer, E.: *Ibid.*, xxxiii, p. 151, 1901.

[3] Cf. Osborne and Jones: *Amer. Journ. of Physiol.*, xxvi, p. 305, 1910.

The constant results obtained, not only in estimating glutaminic acid from casein, but from other proteins, has caused this determination to be regarded as more accurate than that of most of the other products of protein hydrolysis. For this reason the assertion of Skraup[7] that air-dry casein boiled with 33 per cent of sulphuric acid for eighteen hours yielded 16.27 per cent of glutaminic acid and when boiled for six hours with concentrated hydrochloric acid yielded 19.87 per cent appears not to have been generally accepted.

The data presented by Skraup are far from convincing for his estimate of the above percentages is based on the weight of the "crude" glutaminic acid hydrochloride which he isolated. He makes no statement concerning the purity of the product which he weighed and the only evidence that he gives as to the nature of the substance is a chlorine determination made on the *recrystallized* hydrochloride from which, of course, no conclusion can be drawn respecting the degree of purity of the product actually weighed.

The figures given by Skraup, however, agree better with those which we have recently obtained than do those earlier recorded. That casein actually yields at least 15.5 per cent of glutaminic acid seems to be demonstrated by our experience for in five separate determinations we obtained 15.45; 14.82, 14.89; 14.80; and 15.55 per cent. These figures are based on the weight of the perfectly dry hydrochloride which was shown by analysis and melting point to be pure, except for an insignificant quantity of ammonium chloride. It is difficult to attribute these higher results to any other factor than the prolonged hydrolysis to which the casein was subjected in making these determinations although this assumption is not in harmony with Skraup's even higher result, obtained after boiling for only eight hours with concentrated hydrochloric acid. In view of the absence of convincing data we cannot accept Skraup's statements as necessarily contradicting such a conclusion.

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[1] Fischer, E.: *Zeitschr. f. physiol. Chem.*, xxxiii, p. 151, 1901.

[2] Abderhalden: *Ibid.*, xliv, p. 23, 1905.

[3] Osborne and Gilbert: *Amer. Journ. of Physiol.*, xv, p. 333, 1906.

[4] Abderhalden and Funk: *Zeitschr. f. physiol. Chem.*, liii, p. 19, 1907.

[5] Engeland: *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 2962, 1909.

[6] Abderhalden and Langstein: *Zeitschr. f. physiol. Chem.*, lxvi, p. 8, 1910.

[7] Skraup: *Monatsh. f. Chem.*, xxix, p. 791, 1908.

ASPARTIC ACID.

The quantitative determination of aspartic acid by the ester method presents especial difficulties. Our results indicate that much of this trouble is connected with the extraction of the esters with ether. The table on p. 340 shows that we recovered from the products of the first esterification only 10 per cent of the amount which we recovered in all. Thirty per cent remained with the substances insoluble in ether and 60 per cent was recovered from the second esterification. These figures refer only to the relative proportions of the total amount *recovered*; they would be much smaller if related to the total amount actually present.

No estimate of the approximate amount of aspartic acid can therefore be formed, further than that it probably amounts at most to only a few per cent. Until more data are available 1.39 per cent must be accepted the best figure yet recorded.

TYROSINE.

The proportion of tyrosine from casein has been determined many times and most investigators agree in placing it at about 4.5 per cent. Thus Reach[1] found 4.5 per cent; Cohn[2] obtained 3.5 per cent; and Abderhalden and associates[3] on the basis of several determinations give 4.5 per cent. Three separate determinations of tyrosine which we have made after boiling with 25 per cent sulphuric acid for twenty-four, thirty-six, and twenty hours respectively gave 3.9, 3.2, and 3.4 per cent of the moisture- and ash-free casein. Although we made especial efforts to isolate all the tyrosine possible we have not been able to obtain any larger proportion in a condition fit to weigh. Since according to our experience it is impossible to isolate all of the tyrosine by direct crystallization we can accept Abderhalden's figure of 4.50 per cent as probably very nearly correct.

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[1] Reach: *Virchow's Archiv*, clviii, p. 288, 1899.

[2] Cohn: *Zeitschr. f. physiol. Chem.*, xxvi, p. 395, 1899.

[3] Cf. Abderhalden and Langstein: *Zeitschr. f. physiol. Chem.*, lxvi, p. 8, 1910.

AMMONIA.

The proportion of ammonia which casein yields when hydrolysed with strong hydrochloric acid has been determined by so many different investigators with such uniformly agreeing results what we can consider the proportion of this product of hydrolysis to have been determined with accuracy. Thus Henderson[1] found 1.65; Kutscher[2] 1.59; Hart[3] 1.59; Osborne and Harris[4] 1.61; Gümbel[5] 1.60; and VanSlyke[6] 1.63 per cent. The average of these figures, 1.61 per cent, is undoubtedly very nearly correct.

THE BASIC AMINO-ACIDS.

Hart[7] found among the products of decomposition of casein produced by boiling with sulphuric acid and sodium chloride 4.84 per cent of arginine, 2.59 per cent of histidine, and 5.80 and 5.70 per cent of lysine. Osborne, Leavenworth and Brautlecht[8] confirmed these figures for lysine and histidine finding 5.95 and 2..50 per cent respectively but they found only 3.81 per cent of arginine.

[1] Henderson: *Zeitschr. f. physiol. Chem.*, xxix, p. 47, 1900.

[2] Kutscher: *Ibid.*, xxxi, p. 215, 1901.

[3] Hart: *Ibid.*, 1901, xxxiii, p. 347, 1901.

[4] Osborne and Harris: *Journ. Amer. Chem. Soc.*, xxv, p. 323, 1903.

[5] Gümbel: *Beitr. z. chem. Physiol. u. Pathol.*, v, p. 297, 1904.

[6] VanSlyke: *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3170, 1910.

[7] Hart: *Zeitschr. f. physiol. Chem.*, xxxiii, p. 347, 1901.

[8] Osborne, Leavenworth and Brautlecht: *Amer. Journ. of Physiol.*, xxiii, p. 180, 1908.

Two other determinations gave them 3.42 and 3.39 per cent thus leaving some uncertainty respecting the actual amount of this latter substance. A recent estimation of arginine by VanSlyke[1] based on the amount of nitrogen precipitated by phosphotungstic acid under definite conditions, indicated the presence of 4.09 per cent.

Osborne and Harris[2] found in a preparation of very pure casein 3.49 per cent of basic nitrogen according to Hausmann's modified method, but new determinations which we have recently made with likewise very carefully purified casein have given us somewhat lower results, namely, 3.08, 3.13, 3.24 and 3.25 per cent or an average of 3.18 per cent. The nitrogen in the highest results for histidine, arginine, and lysine which Osborne, Leavenworth and Brautlecht found equals 3.07 per cent of the casein, a result in such close agreement with the new determinations of basic nitrogen that it seems probable that their estimations of 2.50 per cent of histidine, 3.81 per cent of arginine and 5.95 per cent of lysine are very nearly correct.

CYSTINE.

Although casein contains about 0.8 per cent of sulphur only a very small part of this appears to belong to cystine. All attempts to isolate cystine as such from casein have failed and the only evidence of its presence is the formation of a quantity of sulphide when casein is decomposed by caustic alkalies which corresponds to 0.1 per cent of sulphur or about 0.50 per cent of cystine if all of this sulphide sulphur originated from cystine.[3]

In this connection mention may be made of the fact that Rubner[4] obtained 0.23 per cent of methyl mercaptan from casein, but this doubtless is a secondary decomposition product of some more complex substance.

We, therefore, have little definite information respecting the nature or amount of the sulphur-containing products yielded by casein.

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[1] VanSlyke: *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3170, 1910.

[2] Osborne and Harris: *Journ. Amer. Chem. Soc.*, xxv, p. 323, 1903.

[3] Cf. Fleitmann: *Ann. d. Chem.*, lxvi, o. 380, 1847; also Osborne: *Journ. Amer. Chem. Soc.*, xxiv, p. 140, 1902.

[4] Rubner: *Arch. f. Hygiene*, xix, p. 136, 1893.

SERINE.

Fischer[1] obtained from casein a small quantity of serine, equal to about 0.5 per cent but considered the amount actually present to be considerably greater.

OXYPROLINE.

Fischer[2] also obtained a minimal quantity of oxyproline from casein which was equal to only 0.23 per cent.

DIAMINOTRIOXYDODECANIC ACID.

Fischer and Abderhalden[3] isolated about 0.75 per cent of a substance from casein to which they gave this name, but the constitution of which they have not yet determined. Several attempts which we have made to detect this substance among the products of decomposition of casein have failed, probably because we used too little casein. The conditions under which we worked, however, were such that we expected to obtain at least some indication of its presence.

TRYPTOPHANE.

No method exists whereby tryptophane can be estimated with any approach to accuracy. The figure given by Abderhalden is to be considered only as minimal. It may well be that tryptophane forms a not inconsiderable part of most of the proteins and that much of the part still unaccounted for may be made up of this amino-acid.

CARBOHYDRATE GROUPS.

The casein which we used for our analysis was practically free from carbohydrate as shown by the exceedingly feeble and transitory Molisch reaction which it gave. No considerable part of the deficit is, therefore, to be attributed to the presence of carbohydrate in the casein.

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[1] Fischer: *Zeitschr. f. physiol. Chem.* xxxix, p. 155, 1903.

[2] Fischer: *Ibid.*

[3] Fischer and Abderhalden: *Ibid.*, xlii, p. 540, 1904.

PHOSPHORUS.

The presence of phosphorus in casein raises the question as to whether it is a constituent of the protein molecule or of some non-protein group united with a protein group as is haematin with globin in haemoglobin or nucleic acid with protein in the nucleins. Such data as are available indicate that the union is other than a salt-like combination of a phosphorus-containing acid with a protein base.

It seems probable that the phosphorus is a constituent of some organic complex and if this shall be shown to be the case a part of the still undetermined part of casein will be accounted for.

SULPHUR.

The fact that casein yields a vey much smaller proportion of sulphide sulphur than do most other proteins when decomposed with strong alkalies shows that most of the sulphur is present in some other form than cystine. Whether or not this sulphur is a constituent of some complex organic radical deserves more study than it has yet received. If this should prove to be the case a further portion of the undetermined part of casein would be accounted for.

It is not improbable that the low summation of analyses of casein directed simply to estimations of amino-acids is caused by the presence of phosphorus and sulphur-containing organic groups of which these two elements form but a small proportion.

If we accept those data which the preceding review has indicated to be the most reliable our present knowledge of the quantitative proportions of the products of hydrolysis of casein can be summarized as follows:

Glycocoll	0.00
Alanine	1.50[1]
Valine	7.20[1]
Leucine	9.35[2]
Proline	6.70[3]
Phenylalanine	3.20[4]
Glutaminic acid	15.55[1]
Aspartic acid	1.39[1]
Cystine	?
Serine	0.50[5]
Tyrosine	4.50[6]
Oxyproline	0.23[5]
Histidine	2.50[7]
Arginine	3.81[7]
Lysine	5.95[7]
Tryptophane	1.50[4]
Diaminotrioxydodecanic acid	0.75[8]
Ammonia	1.61[9]
Sulphur	0.76[10]
Phosphorus	0.85[10]
	67.85

These results show that in spite of all efforts to obtain maximum yields of the several decomposition products of casein the undetermined portion remains relatively large. If we assume that the amino-acids are in polypeptide union with one another and that the ammonia substitutes one hydroxyl of the dibasic acids, the total of the casein thus accounted for is only 57.7 per cent. Assuming that only 70 per cent of the alanine, valine, leucine, proline, and phenylalanine and 50 per cent of the aspartic acid, serine, tryptophane, diaminotrioxydodecanic acid and oxyproline are recovered by the methods used in this analysis[1] the total so estimated as represented by the combined radicals becomes 71.2 per cent of the casein, thus leaving 28.8 per cent still unaccounted for. A similar calculation of the results of the analysis of zein left only 7.3 per cent as correspondingly unaccounted for.

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[1] Osborne and Guest: This paper.

[2] Levene and VanSlyke: This *Journal*, vi, p. 419, 1909.

[3] VanSlyke: *Ber. d. deutsch. chem. Gesellsch.*, xliv, p. 3170, 1910.

[4] Abderhalden: *Zeitschr. f. physiol. Chem.*, xliv, p. 23, 1905.

[5] Fischer: *Ibid.*, xxxix, p. 155, 1903.

[6] Reach: *Virchow's Archiv.*, p. 288, 1899.

[7] Osborne, Leavenworth and Brautlecht: *Amer. Journ. of Physiol.*, xxiii, p. 180, 1908.

[8] Fischer and Abderhalden: *Zeitschr. f. physiol. Chem.*, xlii, p. 540, 1904.

[9] Osborne and Harris: *Journ. Amer. Chem. Soc.*, xxv, p. 323, 1903.

[10] Hammersten: *Zeitschr. f. physiol. Chem.*, vii, p. 227, 1883.

The nitrogen accounted for in this analysis is equal to 63.6 per cent of the total nitrogen, leaving 36.4 per cent unaccounted for, or 5.68 gm. per 100 gm. of casein. The proportion of casein unaccounted for after calculating the amino-acids and ammonia as combined in the protein is 42.5 gm. per 100 to which the above 5.68 gm. belongs. We thus find that the undetermined residue has a mean nitrogen content of 13.1 per cent. A similar calculation of the mean nitrogen content of the undetermined residues of some other proteins gave the following results: for gliadin 13.2, excelsin 14.0, and legumin 14.3 per cent. These calculations, therefore give no evidence that casein differs in constitution to any marked degree from other proteins which contain no phosphorus.

It might be supposed that the phosphorus of the casein was a part of some organic radical which is united with the protein, as, for example, chondroitin sulphuric acid, or nucleic acid, are united in the mucins or nucleins. If this is, in fact, so the organic radical must be one which contains nitrogen in approximately the same proportion as the mono-amino-acids.

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[1] Cf. Osborne and Jones: *Amer. Journ. of Physiol.*, xxvi, p. 305, 1910.

A NEW DECOMPOSITION PRODUCT OF KERATIN WHICH GIVES MILLON'S REACTION.

PRELIMINARY NOTE.

By ROSS AIKEN GORTNER.

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(Received for publication, April 14, 1911.)

The red coloration produced by the interaction of an aromatic phenol and Millon's reagent (mercuric nitrate in dilute nitrous acid) is one of the most characteristic of the color reactions of the proteins. Among the nineteen known compounds which form the protein molecule only one, *p*-oxy-α-amino phenyl propionic acid (tyrosin) is of such a nature as to give a coloration with Millon's reagent and therefore a positive reaction is taken to prove the presence of tyrosin.

In a recent article[1] I have shown that the coloration of the integuments of the meal worm (*Tenebris molitor*) is due to the presence of a tyrosinase, acting upon a chromogen. In many respects this chromogen resembles tyrosin, in that it is not precipitated by phosphotungstic acid or by basic lead acetate and in that it gives a strong Millon's reaction. It differs from tyrosin, however, by being *extremely soluble* so that when evaporation was carried almost to dryness and a few drops of water were added no tyrosin-like crystals could be obtained, neither did any other of the tyrosin tests, excepting the coloration with tyrosinase, produce positive results.

While this was a solitary instance no special attention was paid to it, the supposition being that the chromogen was present in exceedingly small amounts and was probably tyrosin.

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[1] This *Journal*, vii, p. 365, 1910.

In a later study of the pigment of black wool[1] a melanin was isolated by the action of 0.2 per cent sodium hydroxide which was soluble in alkalies, dilute mineral acids and strong acetic and formic acids. Further study of this body has shown that it is probably a *melano-protein, i.e., an acid albumin in which a portion of the protein molecule is so modified as to possess the properties usually ascribed to melanins;* that is, the pigmented portion of the molecule is readily soluble in dilute acid *while combined in the protein molecule,* but, when hydrolyzed by strong mineral acid or acted on by strong alkali, a pigment is obtained which is insoluble in mineral acids.

In my earlier paper, I define melanin as "those dark pigments which occur normally or pathologically in the animal body, skin, hair or feathers," and I still adhere to this nomenclature and call the "melano-protein" a melanin, since it is doubtless in this form that the pigment occurs in the wool.[2]

Approximately 20 grams of this melanin were hydrolyzed by boiling for 30 minutes with fuming hydrochloric acid. The solution was evaporated to dryness on a water-bath, distilled water added and the black melano-humin filtered off. The remainder of the hydrochloric acid was then precipitated by addition of silver sulphate and the sulphuric acid quantitatively removed by baryta. The clear filtrate was concentrated almost to dryness and allowed to crystallize. Then a small amount of cold water was added and the solution filtered from the crystals. These were well washed with cold water and dried *in vacuo* over concentrated sulphuric acid. The dry crystals weighed about 0.3 gram, were of the characteristic tyrosin shape and gave all of the usual tyrosin tests (Millon's, Pyria's, Denigès' and Mörner's and the coloration with tyrosinase). To my surprise, however, the mother liquor still gave Millon's reaction *with undiminished intensity*. Thinking that perhaps hydrolysis was incomplete and tyrosin polypeptides might be present, the solution was diluted to 150 cc. and sulphuric acid added to 25 per cent by weight, and the mixture boiled for 22 hours. The acid was then neutralized with baryta and tyrosin, determined in the usual manner. *No tyrosin was present,* but the Millon's reaction was still very intense. A difference was, however, observed in the reaction, for if a large excess of the reagent were added or if the heating had been too vigorous the coloration did not appear or else was rapidly decolorized.

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[1] Gortner: This *Journal*, viii, p. 341, 1910.

[2] Although I have tested many varieties of keratin, including four samples of human hair, horn, black and white feathers, albino hair and feathers, negro hair, horse hair, black and white wool, etc., "melano-proteins" have been obtained only from black wool, red and brown horse hair and auburn human hair. The detailed report on these pigments, alkali albumins, etc., will shortly be ready for publication.

The solution containing this material was evaporated to dryness on a water bath and insufficient water was added to dissolve the residue. On filtering *the most soluble portion was found to contain the Millon reacting material showing beyond doubt that the substance could not be tyrosin.*

Abandoning hope of obtaining the pure compound from this small quantity of material I hydrolyzed 300 grams of air-dried black wool with two liters of 25 per cent sulphuric acid, boiling the mixture for 24 hours. After separating tyrosin (6.1 grams) in the usual manner, I found the Millon's reaction of apparently undiminished intensity and, although I was able to concentrate the reacting material in *the most soluble* fraction (after 20 grams of leucin (?) had been separated which gave no coloration with the reagent and a third fraction was removed which only gave a faint color). I was, however, unable to obtain the substance in any semblance of purity by this method. It is very evident, however, that some aromatic phenolic body is present in this keratin molecule which is not identical with any of the known products of protein hydrolysis.

I expect to take up the study anew in a short time and with the aid of a Geryk vacuum pump it may be possible to separate this component.

CREATIN AND CREATININ METABOLISM IN DOGS WITH ECK FISTULA.[1]

By NELLIS B. FOSTER and HENRY L. FISHER.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

(Received for publication, April 24, 1911.)

A definite relation between hepatic function and the metabolism of creatin and creatinin was suggested by Mellanby,[2] after he had observed low excretions of creatinin and high eliminations of creatin from individuals suffering of cancer and cirrhosis of the liver. Mellanby attributed the low creatinin excretion in these cases to a depression of the liver function[3] resulting in failure of hepatic transformation of creatin to creatinin. Mellanby mentions other possible explanations, however. Thus he says that the creatin may arise from an increased muscle catabolism incident to carcinomatous toxemia, or that creatin may be a product of the new growth and eliminated in larger quantities on that account.

In carcinoma of the liver there is such marked wasting and cachexia that the question of a relation between hepatic function and creatin excretion is confused by the incidence of other factors. That there is an increased creatin excretion in these cases is certain. We have observed in a case of primary carcinoma of the liver an excretion of more than 1 gram of creatin *per diem*. A high excretion of creatin was also noted in a case of brown atrophy of the liver. Both patients were on a milk diet and there was a steady decrease in weight, to which, at least in part, may be attributed the creatin excretion.

These considerations led us to investigate the relation that the liver may bear to creatin and creatinin excretion in connection with other studies of hepatic function now in progress. The experiments were performed on two dogs. The only means we possess for investigating this point in mammals is by Eck fistula. How completely this operation deprives an animal of the normal hepatic processes is not clear, but we are of the opinion that such deprivation is by no means entire.

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[1] A preliminary report of this work was published in the *Proceedings of the Society for Experimental Biology and Medicine*, viii, p. 33, 1910.

[2] Mellanby: *Journ. of Physiol.*, xxxvi, p. 447, 1908.

[3] Mellanby: *loc. cit.*, p. 481.

The creatin and creatinin metabolism in dogs with Eck fistula has lately been investigated by London and Boljarski,[1] with the following rather remarkable conclusions. (1) The creatin excretion was higher on fast days than on the days when food was given. (2) The addition of creatinin to the food did not increase creatinin excretion. (3) The addition of creatin to the food caused no increased elimination of creatin in the urine, but was followed by a rise in creatinin excretion.

The diet fed to our dogs consisted of unsweetened condensed milk, cracker meal, lard and water. The first dog took his food without apparent relish and never ate the prescribed amount. The constant loss of weight in this dog may be due to his failure to eat rather than to his Eck fistula. The second dog, however, took food well and maintained normal weight.

The accompanying table presents the data collected in this study. The creatin used in these experiments gave no reactions for creatinin or for chloride. From this creatin, creatinin was prepared for us in this laboratory by Dr. S. R. Benedict. The creatin and creatinin were given in the food. The administered amounts were computed from the figures for the content of the one or the other in the available specimens as determined directly by the corresponding Folin method.

A study of the table shows that ingestion of creatinin was followed in each instance by an increased creatinin excretion. In one case there was a rise in creatin elimination after the administration of creatinin. This result was not confirmed, however, and was probably due to some other factor. After creatin ingestion there was no corresponding rise in creatin output; a slight increase in creatinin excretion that was observed thereafter is suggestive but not convincing.

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[1] London and Boljarski: *Zeitschr. f. physiol. Chem.*, lxii, p. 465, 1909.

Data pertaining to experiments on dogs with Eck fistula.

First Dog.

DATE, 1910	BODY WEIGHT	AMOUNT OF URINE	TOTAL N	CREATININ	CREATIN	AMOUNT ADMINISTERED
	kilos	*cc.*	*gram*	*gram*	*gram*	
April 11	11.85	625	3.79	0.209	None	
12	11.81	410	2.41	0.167	0.040	
13	11.45	690	4.50	0.364	0.084	2.33 gm. creatin.
14	11.60	330	3.77	0.251	0.046	
15	11.50	340	2.81	0.219	0.027	
16	11.50	300	2.47	0.135	0.015	
17	11.30	420	5.46	0.399	0.032	
18	11.20	490	2.79	0.172	0.014	
19	11.35	840	6.29	0.756	0.043	0.6 gm. creatinin.
20	11.05	240	2.89	0.166	0.017	
21	10.80	420	4.99	0.318	0.067	1.2 gm. creatin.
22	10.68	550	3.57	0.234	0.063	
23	10.12	780	7.64	0.694	0.208	0.35 gm. creatinin
24	10.07	425	3.62	0.187	0.161	

Second Dog.

July 2	10.67	170	4.76	0.418	0.017	
3	10.35	130	3.06	0.346	0.078	
4	10.85	240	6.92	0.381	0.004	
5	10.65	160	5.87	0.350	0.041	1.11 gm. creatin.
6	10.70	115	2.58	0.253	0.002	
7	10.60	100	2.94	0.288	0.080	1.0 gm. creatin.
8	10.65	430	9.99	0.507	0.009	0.3 gm. creatinin.
9	10.60	180	4.69	0.460	0.000	
10	10.78	135	2.56	0.360	0.008	
11	10.72	160	4.96	0.373	0.076	
12	10.68	320	3.14	0.326	0.014	
13	10.71	175	3.14	0.378	0.004	
14	10.70	190	3.04	0.363	0.017	
15	10.76	420	3.31	0.340	0.008	
16	10.72	355	4.12	0.387	0.161	1.07 gm. creatin.
17	10.65	230	2.77	0.324	0.000	
18	10.68	160	3.89	0.301	0.010	
19	10.67	300	3.03	0.486	0.011	0.5 gm. creatinin.

Both creatin and creatinin appear to act as diuretics. The increase in nitrogen excretion on the days of their ingestion was possibly due to a flushing of excretory substances rather than to a conversion of creatin or creatinin into eliminated nitrogenous derivatives. The disproportion between the urinary nitrogen and the amount of substance administered suggests this explanation. (Compare the data for the 13th and the 21st days; also those for the 19th and the 23d days—first dog.)

We are indebted to Drs. E. H. Poole and H. H. Janeway for their kindness in performing the surgical operations.

A NOTE REGARDING THE PRESENCE OF IODINE IN THE HUMAN PITUITARY.

By W. DENIS.

(*From the Physiological Laboratory of the Tulane Medical School.*)

(Received for publication, April 27, 1911.)

In a recent paper Wells[1] points out that the previous history of patients whose pituitaries are examined for the presence of iodine should always be investigated because the administration of iodides or bandaging with iodoform dressings might lead to a storing of iodine in the hypophysis; and that for this reason the observations of Schnitzler[2] and of Wells[3] that iodine is present in this organ are not conclusive.

In the same paper Wells gives an account of a second series of iodine determinations made by him on human pituitaries, from individuals who had received no iodides or other iodine compounds before death; in these he could detect no trace of iodine, while in an experiment made with three pituitaries from persons who had received iodides before death he was able to detect a trace of iodine which was however too small to permit of quantitative determination.

As it seemed most desirable that further observations regarding this matter should be made I have secured twenty-one human pituitary bodies which have been kept in absolute alcohol for several months before use, they were then dried, the alcohol in which they had been preserved evaporated to dryness in the presence of a small amount of sodium hydrate and the residue so obtained fused with the dried tissue. For the fusions and subsequent extraction Riggs'[4] modification of the Baumann method was used.

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[1] This *Journal*, vii, p. 259.

[2] *Wien. klin. Wochenschr.*, ix, p. 657.

[3] *Journ. of the American Med. Assn.*, xxix, p. 1011.

[4] *Journ. Amer. Chem. Soc.*, xxxi, p. 710.

Sixteen pituitaries taken from patients who had received no iodides before death had, when dried, a total weight of 1.58 grams and on fusion by the above method gave no trace of iodine.

From five hypophyses obtained from the bodies of individuals who had received iodides before death, which had a total weight, when dry, of 0.49 grams I could also obtain no trace of iodine; I am unable to obtain any data as to how long before death the last dose of iodides was administered to the patients from whom these pituitaries were taken.

The material used in this work was taken in the autopsy room of the New Orleans Charity Hospital and was collected for me through the kindness of Drs. F. B. Gurd and M. J. Couret of the Department of Pathology of this University.

THE OXIDATION OF THE AMINO-ACIDS. I. GLYCOCOLL AND CYSTIN.

By W. DENIS.

(From the Physiological Laboratory of the Tulane Medical School.)

(Received for publication, April 27, 1911.)

Considering the importance of the subject comparatively few quantitative data are available concerning the action of oxidizing agents on the amino-acids; it has therefore seemed worth while to investigate the changes produced by the action of potassium permanganate on two of the most widely distributed of the aliphatic amino-acids, viz., glycocoll and cystin.

The oxidation of glycocoll with potassium permanganate has previously been investigated by Engel,[1] by Drechsel[2] and by Halsey[3] who by carrying out the reaction in the presence of an excess of ammonia noted the production of oxamic, oxalic and carbonic acids.

By boiling gelatin with a solution of calcium permanganate Kutscher and Schenck[4] obtained large quantities of oxamic acid and a small amount of oxamid, both of which bodies they consider to be derived from the glycocoll fraction of the gelatin.

OXIDATION OF GLYCOCOLL WITH ALKALINE POTASSIUM PERMANGANATE.

2.4 grams of Kahlbaum's glycocoll dissolved in 100 cc. of water were treated with 300 cc. of a solution containing 45 grams of potassium hydrate (30.0 molecules) and 71.3 grams of potassium permanganate. The mixture was contained in a flask provided with a reflux condenser which was in turn connected with a series of wash bottles containing decinormal hydrochloric acid. On bringing the ingredients together the temperature rose spontaneously to 40° C.; the apparatus was allowed to stand at room temperature for thirty hours, when, to complete the decolorization of the permanganate the flask was warmed in a paraffin bath kept at 120° for five hours. During this time a slow stream of air free from carbon dioxide was drawn through the apparatus, the ammonia given off being collected in the hydrochloric acid; by subsequent titration of the acid the amount of ammonia produced in the reaction was determined.

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[1] *Journ. f. prakt. Chem.*, 1874, p. 847.

[2] *Ber. d. k. Gesell. d. Wissensch; math-naturw. Classe*, Sitzung vom. 21. Juli, 1875.

[3] *Zeitschr. f. physiol. Chem.*, xxv, p. 328.

[4] *Ber. d. d. chem. Gesell.*, xxxvii, p. 2928.

The precipitated manganese dioxide was then removed by filtration and the filtrate divided into two equal parts; to one part was added a slight excess of sulphuric acid and the volatile acids removed by distillation and estimated by subsequent titration of the distillate.

The other portion of the filtrate was placed in the apparatus used for the collection of ammonia and to it was added a slight excess of hydrochloric acid, the carbon dioxide evolved being collected in a series of cylinders filled with a saturated solution of barium hydrate; to get rid of all traces of carbon dioxide, the acid liquid was heated for five hours in an oil bath kept at 120°–125° C., a slow current of air freed from carbon dioxide being meanwhile drawn through the apparatus. At the end of this time the barium carbonate formed was rapidly filtered off, washed with boiling water, dried at 110° and weighed.

In the liquid from which the carbon dioxide had been removed oxalic acid was determined by precipitation with calcium acetate, the solution having first been neutralized with ammonium hydroxide. After removal of the calcium oxalate by filtration the liquid was evaporated to small volume: 25 grams of potassium hydrate were then added and the solution boiled for six hours in a flask provided with a reflux condenser in order to obtain any oxamic acid present in the form of calcium oxalate.

In a second experiment the oxalic acid was isolated for purposes of identification by extraction with ether and was obtained in characteristic crystals melting at 99° C.

By proceeding in the manner just outlined I obtained from the above-mentioned 2.4 grams of glycocoll, 0.96 gram of carbon dioxide, 0.50 gram of ammonia, 2.00 grams of oxalic acid, no oxamic acid, and volatile acids equivalent to only 4.00 cc. of decinormal sodium hydroxide. Subsequent tests showed that this small amount of volatile acid consisted entirely of nitric acid formed from ammonia by the oxidizing action of the permanganate. These results would seem to indicate that in the oxidation of glycocoll by permanganate in strongly alkaline solution 69 per cent of the amino-acid is transformed according to the following equation:

$$NH_2.CH_2.COOH + O_2 \rightarrow NH_3 + (COOH)_2$$

Whereas the remaining 33 per cent is oxidized according to the equation:

$$NH_2.CH_2.COOH + O_2 \rightarrow NH_3 + HCOOH + CO_2$$

Under the condition of the experiment the formic acid then further reacts to give carbon dioxide and water.

It is also easily conceivable that the above mentioned 33 per cent of amino-acid may be oxidized as follows:

$$2\,NH_2.CH_2.COOH + O_2 \rightarrow 2\,NH_3 + 2\,CO_2 + 2HCHO$$

with subsequent oxidation of the formaldehyde to formic acid which in turn is transformed into carbon dioxide and water.

The results obtained in the following experiment point to the former reactions as probably furnishing the true explanation, although it is easily conceivable that the formic acid found may have resulted from the oxidation of the previously formed formic aldehyde.

On oxidizing in the cold 0.5 gram of glycocoll dissolved in 25 cc. of water with 60 cc. of a mixture containing 3.3 grams of potassium hydrate and 0.62 gram of potassium permanganate, decolorization of the permanganate took place within twenty minutes; after filtration the liquid was tested for formic aldehyde by Leaches' modification of the Hehner test and by the resorcin sulphuric acid test but only negative results were obtained. Formic acid was shown to be present by the reaction with mercuric salts. With Schiff's reagent the liquid gave a positive reaction, while indication of the presence of a trace of glyoxylic acid could be obtained by the application of the tryptophane test. The presence of glyoxylic acid in the mixture of partial oxidation products of glycocoll makes it necessary to consider two possibilities to account for the formation of oxalic acid from this substance.

Dakin[1] has shown that by the oxidation of glycocoll and various other substituted acetic acids with hydrogen peroxide large quantities of glyoxylic acid are formed together with ammonia, formaldehyde and formic and carbonic acids. As is well known glyoxylic acid is rapidly transformed into oxalic acid, *by the action* of alkaline permanganate. It is therefore easily conceivable that the oxalic acid obtained is formed by a secondary reaction from the glyoxylic acid first produced.

On the other hand it has been shown by Engel, Drechsel and later by Halsey that when the oxidation of glycocoll by permanganate is conducted in the presence of an excess of ammonium hydrate large quantities of oxamic acid are formed; by the method of procedure which I have used it is possible that the first product of oxidation in this case also may be oxamic acid which is at once decomposed into oxalic acid and ammonia by the action of the large excess of alkali used in the oxidizing mixture.

In this connection the work of Lusk[2] on the fate of the amino-acids in the animal body is of interest; on the administration of glycocoll to diabetic dogs he finds the amino-acid to be entirely eliminated in the urine as sugar; from this and other experiments he concludes that the process of deamination of glycocoll in the body is one of hydrolysis and not of oxidation, the intermediate substances formed in the transformation of glycocoll into glucose being in his opinion glycollic acid and glycolaldehyde.

$$NH_2.CH_2.COOH + H_2O \rightarrow CH_2OH.COOH + NH_3$$

It is conceivable as a third possibility that glycollic acid may be the intermediate body from which the oxalic acid resulting from the oxidation of glycocoll is formed. On account of the ease with which glycollic acid is transformed into oxalic acid by oxidizing agents there is little hope of obtaining qualitative proof of its presence by the method of treating the amino-acid with an insufficient amount of oxidizing agent; my own experiments made along this line have met with negative results.

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[1] This *Journal*, i, pp. 171, 271.

[2] *Journ. Amer. Chem. Soc.*, xxxii, p. 673.

PREPARATION OF CYSTIN.

The cystin used in the experiments given below was prepared from human hair and from feathers by the following method which, while it does not give the large yields to be obtained by the original procedure of Mörner, allows one to obtain with a small amount of labor a pure preparation of the amino-acid.

The fat-free hair or feathers were placed in a flask containing twice their weight of 20 per cent hydrochloric acid, the flask provided with a reflux condenser was heated on a sand bath for ten to fourteen hours. The liquid was then poured into an evaporating dish and a large part of the hydrochloric acid removed by evaporation on the water bath. While still hot, crystals of sodium acetate were added to the liquid until congo red paper no longer indicated the presence of free mineral acid. This method of neutralising the mineral acid, which has recently been suggested by Folin[1] is accompanied with a much smaller loss of time and material than when this process is effected with a strong solution of caustic alkali in the way usually recommended. The mixture is then allowed to stand at laboratory temperature for from four to five days, after which the precipitate is filtered off, well washed with cold water, dissolved in 4 per cent hydrochloric acid and boiled with bone black; after filtration the bone black is boiled with two portions of 4 per cent hydrochloric acid, the various filtrates united, evaporated to small volume in the water bath and the cystin precipitated with a hot solution of sodium acetate. An equal volume of acetone is then added and the mixture allowed to stand at laboratory temperature for two days, when the cystin is filtered off and well washed with cold water. By this method I obtained from turkey feathers a yield of 2.5 per cent of cystin, from chicken feathers, 3.2 per cent, from the feathers of the wild duck, 4.00 per cent, and from human hair, 5.5 per cent.

A word regarding the advisability of evaporating down hydrochloric acid solutions of cystin on the water bath may not be out of place at this point. In the preparation of cystin most authors[2] have advised the removal of excess of hydrochloric acid by distillation at reduced pressure.

The only quantitative results concerning the action of dilute acids on this amino-acid with which I am familiar are those of Mörner[3] who heated cystin dissolved in 10 per cent hydrochloric acid on the water bath for 109 hours; at the end of this time the specific rotation of the material which had been at the beginning of the experiment −223° had fallen to −134° while only 82 per cent of the cystin could be recovered from the solution; a trace of sulphuric acid was present but no hydrogen sulphide.

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[1] This *Journal*, viii, p. 9.

[2] Mörner: *Zeitschr. f. physiol. Chem.*, xxviii, p. 695; Abderhalden and Teruchi: *Ibid.*, xlviii, p. 528; Mathews: This *Journal*, vi, p. 21.

[3] Mörner: *Zeitschr. f. physiol. Chem.*, xxxiv, p. 208.

The following experiments were undertaken with the idea of obtaining some data as to the ease with which cystin may be decomposed with boiling mineral acids.

The method of procedure was as follows:

9.5935 grams of cystin were dissolved in 100 cc. of 2 per cent hydrochloric acid and the solution then diluted with water to 250 cc. Aliquot portions of this solution were heated on the steam bath with various concentrations of hydrochloric acid in a flask provided with a reflux condenser.

At the end of the period of heating the hydrochloric acid was neutralized with sodium acetate, to the solution was added twice its volume of 95 per cent alcohol and the whole allowed to stand at laboratory temperature for four days, at the end of which time the precipitated cystin was filtered off on a tared Gooch crucible, well washed with cold water and with 95 per cent alcohol and dried at 95° C.

	Cystin Recovered	
	gram	*per cent*
(1) 25 cc. cystin solution precipitated as soon as made..	0.9065	95.2
(2) 25 cc. cystin solution + 50 cc. 39 per cent Hcl, heated for 25 hours	0.8395	88.3
(3) 25 cc. cystin solution + 25 cc. 39 per cent Hcl, heated for 25 hours	0.8321	87.5
(4) 25 cc. cystin solution + 25 cc. 39 per cent Hcl, heated for 5 hours	0.8467	89.1
(5) Same as (4) except that solution was evaporated in open dish on water bath for 4 hours	0.8510	99.2
(6) 20 cc. cystin solution stood in stoppered flask in laboratory for 37 days	0.6807	89.5

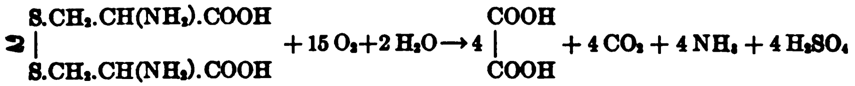
No hydrogen sulphide was formed in any of the above experiments but traces of sulphuric acid could be detected in all except (1).

OXIDATION OF CYSTIN WITH ALKALINE POTASSIUM PERMANGANATE.

To 2.1 grams of cystin suspended in 25 cc. of water were added 400 cc. of a solution containing 5.5 grams of potassium hydroxide (10 molecules) and 11.6 grams of potassium permanganate; this mixture was allowed to stand at room temperature for thirty-six hours, at the end of which time the permanganate was entirely decolorized. Ammonia was quantitatively determined by the method previously described, the liquid was then filtered and the filtrate and washings mixed and divided into three parts. To one portion was added a slight excess of hydrochloric acid and carbon dioxide determined as for glycocoll; this portion was then evaporated to small volume on the water bath and oxalic acid extracted with ether; in another portion sulphuric acid was determined by precipitation with barium chloride in the presence of an excess of hydrochloric acid, while to the third part was added a slight excess of sulphuric acid and volatile acids distilled off and estimated by titration.

By these methods I obtained from the above-mentioned 2.1 grams of cystin 0.23 gram of ammonia, 0.90 gram of carbon dioxide, 1.13 grams of oxalic acid, 1.28 grams of sulphuric acid, traces of free sulphur, and volatile acids equivalent to 20.0 cc. of $\frac{N}{10}$ potassium hydroxide. The oxalic acid found was identified by its melting point, 99° C., and by the preparation of its calcium salt.

The distillate containing volatile acids was neutralized with potassium carbonate and evaporated to dryness on the water bath, the residue was then repeatedly extracted with absolute alcohol to separate potassium acetate and potassium nitrate by the method of Juan.[1] 0.085 gram of potassium acetate, equivalent to 0.051 gram of acetic acid, was obtained, the residue insoluble in absolute alcohol gave positive results with the usual qualitative tests for the determination of nitrates and consisted of potassium nitrate derived from the ammonia by the action of permanganate. From these results it would seem that by oxidation of cystin with permanganate in strongly alkaline solution the largest part of the material is transformed according to the equation,



$$2\begin{array}{l}S.CH_2.CH(NH_2).COOH\\ |\\ S.CH_2.CH(NH_2).COOH\end{array} + 15\,O_2 + 2\,H_2O \rightarrow 4\begin{array}{l}COOH\\ |\\ COOH\end{array} + 4\,CO_2 + 4\,NH_3 + 4\,H_2SO_4$$

As an intermediate product in the formation of oxalic acid lactic acid with subsequent transformation into pyruvic acid would seem most probable.

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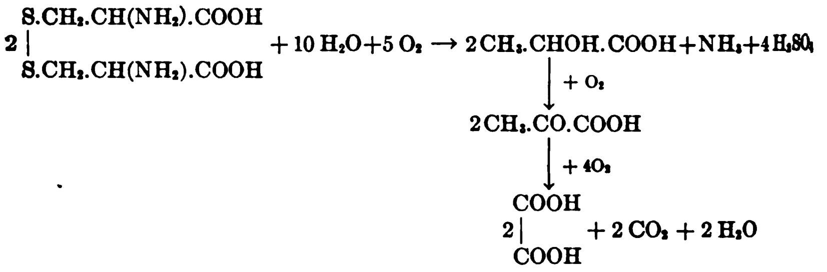
[1] *Amer. Chem. Journ.*, xliii, p. 1.



$$2\left|\begin{array}{l}S.CH_2.CH(NH_2).COOH\\ S.CH_2.CH(NH_2).COOH\end{array}\right. + 10\,H_2O + 5\,O_2 \rightarrow 2\,CH_3.CHOH.COOH + NH_3 + 4\,H_2SO_4$$

$$\downarrow + O_2$$

$$2\,CH_3.CO.COOH$$

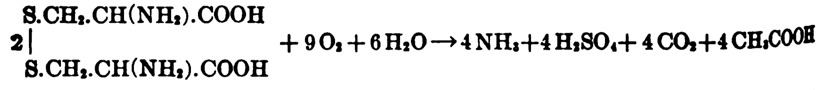
$$\downarrow + 4O_2$$

$$2\left|\begin{array}{l}COOH\\ COOH\end{array}\right. + 2\,CO_2 + 2\,H_2O$$

Beilstein[1] has shown that calcium lactate when oxidized with an insufficient quantity of potassium permanganate gives a fair amount of pyruvic acid, while it was found by the author[2] that pyruvic acid when treated with alkaline permanganate is converted almost quantitatively into molecular amounts of oxalic acid and carbon dioxide.

As an attempt to test the truth of the above statements the following experiment was performed. 1.0 gram of cystin suspended in 25 cc. of water was treated in the cold with 75 cc. of a solution containing 2.32 grams of potassium permanganate and 4.2 grams of potassium hydrate; decolorization of the permanganate took place within fifteen minutes. On filtering off the precipitated manganese dioxide the filtrate was found to give a positive reaction with Schiff's and with Tollens' reagents. The remainder of the filtrate made slightly acid with hydrochloric acid was extracted with ether; on removal of the ether by distillation a small quantity of an acid oil was left in the distilling flask, an aqueous solution of which reduced Fehling's solution when heated, and gave a small amount of a hydrazone melting when crude at 175° C.

From the detection of a small amount of acetic acid among the products of oxidation it is evident that a portion of the cystin was transformed into ammonia and acetic, carbonic and sulphuric acids either by the following reaction:



$$2\left|\begin{array}{l}S.CH_2.CH(NH_2).COOH\\ S.CH_2.CH(NH_2).COOH\end{array}\right. + 9\,O_2 + 6\,H_2O \rightarrow 4\,NH_3 + 4\,H_2SO_4 + 4\,CO_2 + 4\,CH_3COOH$$

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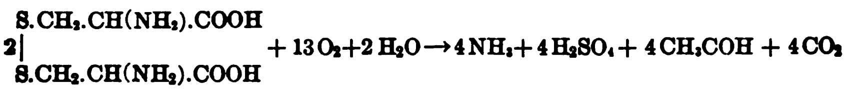
[1] *Ber. d. d. chem. Ges.*, xvii, p. 840.

[2] *Amer. Chem. Journ.*, xxxviii, p. 569.

or by way of a primary formation of acetaldehyde, which would then be immediately oxidized by the alkaline permanganate into oxalic and acetic acids.[1]



$$2\left|\begin{matrix}\mathrm{S.CH_2.CH(NH_2).COOH}\\ \\ \mathrm{S.CH_2.CH(NH_2).COOH}\end{matrix}\right. + 13\,O_2 + 2\,H_2O \rightarrow 4\,NH_3 + 4\,H_2SO_4 + 4\,CH_3COH + 4\,CO_2$$

Somewhat less than the calculated amount of ammonia was collected. Whether this deficiency is due to the retention of nitrogen in combination in some compound which I have failed to isolate or whether it be entirely due to the oxidation of a part of the ammonia split off to nitric acid and nitrogen, which reaction has been shown to occur by Herschkowpsch[2], it is impossible to say.

The amount of sulphuric acid precipitated as barium sulphate is also somewhat below the amount demanded by theory if the calculation be based on the assumption that both sulphur atoms in the cystin molecule are split off as hydrogen sulphide, which compound is subsequently oxidized to sulphuric acid. The invariable appearance of particles of free sulphur will however readily account for this discrepancy.

In conclusion I wish to express my thanks to Prof. Gustav Mann who has furnished me throughout the course of this work with much helpful criticism, and many valuable suggestions.

SUMMARY.

1. Glycocoll on complete oxidation with alkaline potassium permanganate was found to yield oxalic acid, carbon dioxide, ammonia, and nitric acid. When glycocoll was treated with an insufficient amount of the oxidizing agent traces of formic acid and of glyoxylic acid could be detected.
2. A modification of the existing methods for the preparation of cystin is described.
3. Cystin on complete oxidation with alkaline permanganate yielded oxalic, sulphuric, carbonic, acetic and nitric acids together with ammonia and free sulphur. By treatment of the amino-acid

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[1] Denis: *Amer. Chem. Journ.*, xxxviii, p. 569.

[2] *Zeitschr. f. physikal. Chem.*, lx, p. 93.

with an insufficient amount of the oxidizing agent traces of pyruvic acid could be demonstrated.

Further experiments are in progress in this laboratory dealing with the action of permanganate without alkali on cystin and glycocoll and also on other aliphatic and aromatic amino-acids.

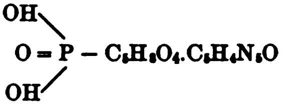
THE ACTION OF GASTRO-INTESTINAL JUICES ON NUCLEIC ACIDS.

By P. A. LEVENE and F. MEDIGRECEANU.

(From the Laboratories and the Hospital of the Rockefeller Institute for Medical Research, New York.)

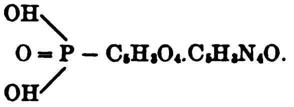
(Received for publication, April 28, 1911.)

On the basis of the recent work of Levene and Jacobs, nucleic acids may be regarded as mono- or polynucleotides. Under the name nucleotides are designated compounds consisting of phosphoric acid conjugated with a complex composed of a carbohydrate and a base. Inosinic, or guanylic acids may be considered as types of such nucleotides. In chemical terms the composition of these substances may be expressed in the following way:



$$(OH)_2O=P-C_5H_8O_4.C_5H_4N_5O$$

or



$$(OH)_2O=P-C_5H_8O_4.C_5H_3N_4O.$$

In the course of metabolism the simple as well as the complex nucleic acids undergo complete disintegration. The part played in this process by the gastro-intestinal tract has not been known until very recently. The number of investigations on the direct action of the gastro-intestinal juices is small, and the greatest part of the work was done at a time when the chemical structure of the nucleic acids was still unknown. A review of the literature up to the article of London and Schittenhelm is given in the monograph of Brugsch and Schittenhelm.[1]

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[1] Brugsch und Schittenhelm: *Der Nucleinstoffwechsel und seine Störungen*, Gustav Fischer, Jéna, 1910.

The results of the recent work of London and Schittenhelm[1] recorded in their last article lead them to the view that nucleic acids are not digested nor absorbed by the stomach. In the intestines, however, nucleic acids undergo decomposition either complete, with the liberation of purin bases, or partial, leading to the formation of nucleosides. The experiments of London and Schittenhelm were performed on dogs with fistulae in different parts of the intestine. The substances employed in their experiments were the yeast and the thymus nucleic acids.

The evidence for the assumption of the formation of nucleosides in their experiments was indirect. And although the views of these writers may be perfectly correct, nevertheless it could not be regarded as firmly established by experiment.

In a previous communication[2] the present writers pointed out that the mechanism of the dissolution of the nucleic acid molecule can be elucidated most conveniently by a comparative study of the dissolution of the smaller fragments of the molecule. It was also pointed out that the changes in the optical rotation of the solution of the nucleic acid in a general way may serve as an indicator of the extent of the cleavage suffered by the nucleic acid.

The plan of the present investigation was based on these considerations.

The substances employed in the experiments (given in increasing degree of complexity) were the following: Inosin, cytidin, guanylic acid, pyrimidin nucleotides, yeast nucleic acid, and thymus nucleic acid.

The digestive juices were kindly furnished to us by Prof. I. P. Pawlow of St. Petersburg, and we take this occasion to acknowledge our appreciation of his courtesy.

All the juices contained their proteolytic enzymes or zymogens. Thus, the gastric juice caused the usual digestion of a beef powder, but failed to cause the cleavage of glycyltryptophan. The pancreatic juice was inactive when allowed to act on the protein or the peptid, but showed the usual activity on beef powder, and on glycyltryptophan after the addition of a small quantity of enterokinase. The intestinal juice brought about a very intense cleavage of glycyltryptophan.

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[1] London und Schittenhelm: *Zeitschr. f. physiol. Chemie*, lxx, p. 10, 1911.

[2] Levene and Medigreceanu: This *Journal*, ix, p. 65, 1911.

The analytical methods employed in course of the investigation will be referred to in the experimental part. The results of the experiments were the following.

Inosin remained unchanged through the action of neutralized gastric juice, pancreatic juice and intestinal juice; also by the combined action of pancreatic and intestinal juices.

Guanosin behaved similarly to inosin.

Cytidin behaved similarly to the previous substances.

Guanylic Acid under the influence of either gastric and pancreatic juice remained unchanged. Under the influence of intestinal juice the following changes occurred: The clear solution of the sodium salt of nucleic acid and the enzyme became turbid on standing. The turbidity ended in the formation of a crystalline precipitate. Simultaneously with the precipitate formation was noted a marked diminution in the optical rotation of the supernatant solution. The precipitate dissolved by the aid of a 10 per cent solution of sodium hydrate possessed a higher optical activity than the original solution of the nucleic acid. The filtrate contained free phosphoric acid which was absent in the original solution. The solution did not reduce Fehling's solution on boiling, but did so after previous hydrolysis with mineral acid.

The chemical reaction that took place under the influence of the enzyme is obvious from this description. It consists, in the detachment of the phosphoric acid from the guanosin. The crystalline precipitate formed on digestion consisted of pure guanosin. This makes evident the reason of the observation that the precipitate possessed a higher rotatory power than the solution of the original guanylic acid.

Pyrimidin Nucleotides. Pyrimidin nucleotides remained unaltered under the influence of neutralized gastric juice or of pancreatic juice. Under the influence of intestinal juice the optical rotation of the solution did not alter perceptibly. During digestion the solution developed free phosphoric acid; though not in the same proportion as a similar solution of guanylic acid. The product of digestion did not reduce Fehling's solution.

Thus, the impression is gained that the pyrimidin nucleotides are decomposed through the action of intestinal juice into phosphoric acid and pyrimidin complexes. However, the reaction takes place with lower intensity than in a similar solution of guanylic acid.

Yeast Nucleic Acid. Under the influence of neutralized gastric juice and of pancreatic juice the solution of the sodium salt of this acid suffered little change in its physical or chemical properties. Under the influence of intestinal juice the following changes were noted: 1. A very rapid fall of the magnitude of the optical rotation of the solution. 2. The nucleic acid could no longer be precipitated from its solution by glacial acetic acid. 3. The solution contained free phosphoric acid but showed no reduction of Fehling's solution on boiling. The reduction was very pronounced after preliminary hydrolysis with mineral acids. It is obvious from this that the following chemical changes took place in the nucleic acid: 1. A striking alteration in its solubility. While the original nucleic acid was insoluble in glacial acetic acid, the solution resulting from the action of intestinal juice was readily soluble in that reagent. It is possible that this change was due to the cleavage of the original nucleic acid into mononucleotides. 2. The cleavage of the nucleotides into phosphoric acid and the organic complexes. These complexes, however, remained intact.

Thymus Nucleic Acid. Under the influence of neutralized gastric juice and of pancreatic juice, no alteration of the sodium salt of the original nucleic acid took place. Under the influence of intestinal juice the following changes occurred: While the original solution formed after action of hydrochloric acid a precipitate of the free nucleic acid, such a precipitate did not form after the action of the intestinal juice. Simultaneously there occurred a very definite decline in the optical rotation of the original solution. This, however, took place at a much lower intensity than a similar solution of yeast nucleic acid. The solution showed the presence of free phosphoric acid; did not reduce Fehling's solution on boiling but did so after a previous hydrolysis with mineral acids. Thus the changes occurring in the thymus nucleic acid are apparently the same as in the yeast nucleic acid, but they are brought about at a considerably lower intensity.

EXPERIMENTAL PART.

The optical measurements were made in the manner described in the communication on nucleases.[1] Care was exercised to allow the nucleic acid solutions to cool to room temperature, since the observation had been made that the magnitude of the optical rotation of these solutions was affected by the temperature.

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[1] Levene and Medigreceanu: This *Journal*, ix, p. 65, 1911.

Phosphoric acid estimations were made in the following manner: To the neutral solutions molybdic solution was added as long as a precipitate formed. This was removed by filtration. The filtrate was treated with ammonia water and acidulated with nitric acid. In the presence of free phosphoric acid the yellow precipitate formed.

The presence of free sugar was tested by means of Fehling's solution. In the presence of purin bases a white precipitate of the cuprous salts of the purins formed.

INOSIN EXPERIMENTS.

Experiment with Neutral Gastric Juice.

A. *In neutral phosphate solution (1 per cent).*

III, 28, '11. Enzyme solution, 1 cc.
Inosin solution, 4 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	24 hrs.	144 hrs.
Experiment:	−0.76	−0.76	−0.76	−0.76
Control:	0.00	0.00	0.00	0.00

B. *In acid solution.*

III, 28, '11. Inosin, 0.2 gm. in
Acid gastric juice, 5 cc.

Control: Inosin, 0.2 gm. in
Hydrochloric acid, 0.5 per cent, 5 cc.

	10 min.	12 hrs.	36 hrs.	94 hrs.	190 hrs.
Experiment:	−0.76	−0.75	−0.65	−0.50	−0.31
Control:	−0.76	−0.75	−0.60	−0.50	−0.32

Experiments with Pancreatic Juice.

In neutral phosphate solution (1 per cent).

A. *Unactivated Pancreatic Juice.*

III, 28, '11. Enzyme solution, 0.5 cc.
Inosin solution, 4 per cent, 3.5 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	24 hrs.	168 hrs.
Experiment:	−0.87	−0.87	−0.87
Control:	−0.04	−0.04	−0.04

B. *Activated pancreatic juice.*

III, 29, '11. Pancreatic juice, 1 cc.
Intestinal juice, 0.1 cc.
Inosin solution, 4 per cent, 3.5 cc.

Control: Enzyme solution, 1.1 cc.
Phosphate solution, 3.5 cc.

	10 min.	24 hrs.	168 hrs.
Experiment:	−0.76	−0.75	−0.75
Control:	−0.08	−0.08	−0.08

Experiments with Intestinal Juice.

In neutral phosphate solution (1 per cent).

Experiment I.

III, 28, '11. Enzyme solution, 0.1 cc.
Inosin solution, 4 per cent, 3.9 cc.

Control: Enzyme solution, 0.1 cc.
Phosphate solution, 3.9 cc.

	10 min.	24 hrs.	144 hrs.
Experiment:	−0.94	−0.94	−0.94
Control:	0.00	0.00	0.00

Experiment II.

III, 29, '11. Intestinal juice, 1 cc.
Inosin solution, 4 per cent, 3 cc.

Control: Intestinal juice, 1 cc.
Phosphate solution, 3 cc.

	10 min.	24 hrs.	72 hrs.	96 hrs.	168 hrs.
Experiment:	−0.73	−0.70	−0.70	−0.69	−0.70
Control:	−0.03	−0.03	−0.03	−0.03	−0.03

The sugar test (Fehling's solution) gave in all the above experiments negative results.

CYTIDIN EXPERIMENTS.

Experiment with Neutral Gastric Juice.

III, 28, '11. Gastric juice, 1 cc.
Cytidin solution, 3 cc.

Control: Gastric juice, 1 cc.
Phosphate solution, 3 cc.

	10 min.	24 hrs.	96 hrs.
Experiment:	+0.20	+0.19	+0.19
Control:	0.00	0.00	0.00

Experiment with Acid Gastric Juice.

III, 28, '11. Gastric juice, 1 cc.
Cytidin solution, 3 cc.

Control: Hydrochloric acid, 0.5 per cent, 1 cc.
Cytidin solution, 3 cc.

	10 min.	24 hrs.	96 hrs.
Experiment:	+0.19	+0.19	+0.19
Control:	+0.18	+0.18	+0.18

Experiment with Activated Pancreatic Juice.

III, 29, '11. Pancreatic juice, 1 cc.
Intestinal juice, 0.1 cc.
Cytidin solution, 3 cc.

Control: Pancreatic juice, 1 cc.
Intestinal juice, 0.1 cc.
Phosphate solution, 3 cc.

	10 min.	24 hrs.	120 hrs.
Experiment:	+0.52	+0.52	+0.52
Control:	−0.08	−0.08	−0.08

Experiment with Intestinal Juice.

IV, 1, '11. Intestinal juice, 0.5 cc.
Cytidin solution, 3.5 cc.

Control: Intestinal juice, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	72 hrs.	96 hrs.	120 hrs
Experiment:	+0.63	+0.63	+0.60	cloudy
Control:	−0.02	−0.02	−0.02	−0.02

None of the solutions in the cytidin experiments reduced Fehling's solution (at the end of the experiment).

GUANYLIC ACID EXPERIMENTS.

In physiological NaCl solution.

Experiment with Neutral Gastric Juice.

IV, 3, '11. Gastric juice, 1 cc.
Sodium guanylate solution, 5 per cent, 3 cc.

Control: Gastric juice, 1 cc.
Salt solution, 3 cc.

	10 min.	24 hrs.	120 hrs.
Experiment:	−0.31	−0.31	−0.31
Control:	0.00	0.00	0.00

Experiment with Acid Gastric Juice.

IV, 3, '11. Gastric juice, 1 cc.
Sodium guanylate solution, 3 cc.

Control: Gastric juice, 1 cc.
Salt solution, 3 cc.

	10 min.	24 hrs.	120 hrs.
Experiment:	−0.30	−0.29	−0.29
Control:	0.00	0.00	0.00

Experiment with Unactivated Pancreatic Juice.

IV, 3, '11. Pancreatic juice, 1 cc.
Sodium guanylate solution, 3 cc.

Control: Pancreatic juice, 1 cc.
Salt solution, 3 cc.

	10 min.	24 hrs.	120 hrs.
Experiment:	−0.33	−0.32	−0.32
Control:	−0.08	−0.08	−0.08

Experiment with Activated Pancreatic Juice.

IV, 3, '11. Pancreatic juice, 1 cc.
Intestinal juice, 1 drop.
Sodium guanylate solution, 5 per cent, 3 cc.

Control (1): Pancreatic juice, 1 cc.
Intestinal juice, 1 drop (see cytidin experiment).
Salt solution, 3 cc.

Control (2): Intestinal juice, 1 drop.
Sodium guanylate solution, 3 cc.
Salt solution, 1 cc.

	10 min.	24 hrs.	120 hrs.
Experiment:	−0.33	−0.30	−0.29
Control (2):	−0.31	−0.29	−0.29

In all the above experiments with guanylic acid, the free phosphoric acid test as well as the free sugar test (Fehling's solution) were negative (at the end of the experiment).

EXPERIMENT WITH INTESTINAL JUICE.

IV, 3, '11. Intestinal juice, 0.5 cc.
Sodium guanylate solution, 3 cc.
Salt solution, 0.5 cc.

Control: Intestinal juice, 0.5 cc.
Salt solution, 3.5 cc.

	10 min.	16 hrs.	40 hrs.	96 hrs.
Experiment:	−0.33	cloudy	−0.07	−0.07
Control:	0.00	0.00	0.00	0.00

Free phosphoric acid present. Did not reduce Fehling's solution.

A precipitate was formed consisting of characteristic guanosin crystals. A 2.5 per cent solution of the air-dry precipitate in 10 per cent NaOH showed a rotation of −0.72 in a 5 cm. long observation tube. With the original guanylic acid under the same conditions the rotation was only −0.40.

PYRIMIDIN NUCLEOTIDE EXPERIMENTS.

In physiological NaCl solution.

EXPERIMENT WITH NEUTRAL GASTRIC JUICE.

III, 28, '11. Gastric juice, 1 cc.
Nucleotide solution, 3 cc.

Control: (See Inosin experiments.)

10 min.	24 hrs.	144 hrs.
+0.42	+0.42	+0.42

EXPERIMENT WITH ACID GASTRIC JUICE.

III, 28, '11. Gastric juice, 1 cc.
Nucleotide solution, 3 cc.

Control (1): Gastric juice, 1 cc. (see Inosin experiments).
Salt solution, 3 cc.

Control (2): Hydrochloric acid, 0.5 per cent, 1 cc.
Nucleotide solution, 3 cc.

	10 min.	24 hrs.	48 hrs.	72 hrs.
Experiment:	+0.58	0.58	+0.57	dark
Control (2):	+0.58	+0.58	+0.56	+0.55

EXPERIMENT WITH UNACTIVATED PANCREATIC JUICE.

III, 31, '11 Pancreatic juice, 1 cc.
Nucleotide solution, 3 cc.

Control: Pancreatic juice, 1 cc.
Salt solution. (See Inosin experiment).

10 min.	24 hrs.	96 hrs.	120 hrs.
+0.42	+0.43	+0.42	dark

EXPERIMENT WITH ACTIVATED PANCREATIC JUICE.

III, 31, '11. Pancreatic juice, 1 cc.
Intestinal juice, 1 drop.
Nucleotide solution, 3 cc.

Control: Pancreatic juice, 1 cc.
Intestinal juice, 1 drop (see cytidin experiment).
Salt solution, 3 cc.

10 min.	96hrs.	120 hrs.
+0.42	+0.40	dark

The tests for free phosphoric acid and free sugar (Fehling's solution) were negative at the end of all the above nucleotide experiments.

EXPERIMENT WITH INTESTINAL JUICE.

IV, 11, '11. Intestinal juice, 0.5 cc.
Nucleotide solution, 3.5 cc.

Control: Intestinal juice, 0.5 cc. (see guanylic acid experiment).
Salt solution, 3.5 cc.

10 min.	24 hrs.	48 hrs.	72 hrs.
+0.19	+0.17	+0.13	+0.13

Free phosphoric acid present. Did not reduce Fehling's solution.

YEAST NUCLEIC ACID EXPERIMENTS.

In physiological NaCl solution. Reading at the room temperature

Experiment with Neutral Gastric Juice.

IV, 1, '11. Gastric juice, 1 cc.
Sodium nucleinate solution, 10 per cent, 3 cc.

Control (1): Gastric juice, 1 cc. (See guanylic acid experiment).
Salt solution, 3 cc.

Control (2): Sodium nucleinate, 10 per cent.

	10 min.	24 hrs.	72 hrs.	144 hrs.	168 hrs.
Experiment:	+4.25	+3.50	+3.20	+3.00	+3.00
Control (2):	+5.75	+5.60	+5.55	+5.40	+5.35

Free phosphoric acid absent. Did not reduce Fehling's solution. Glacial acetic acid gave a heavy precipitate.

Experiment with Unactivated Pancreatic Juice.

III, 28, '11. Pancreatic juice, 0.5 cc.
Sodium nucleinate solution, 6 per cent, 3.5 cc.

Control (1): Pancreatic juice, 0.5 cc.
Salt solution, 3.5 cc.

Control (2): Sodium nucleinate, 6 per cent.

	10 min.	24 hrs.	48 hrs.	96 hrs.	144 hrs.
Experiment:	+2.50	+2.20	+2.10	+2.02	+2.00
Control (1):	−0.04	−0.04	−0.04	−0.04	−0.04
Control (2):	+2.90	+2.80	+2.80	+2.70	+2.70

Free phosphoric acid absent. Did not reduce Fehling's solution. Glacial acetic acid gave a heavy precipitate.

Experiment with Activated Pancreatic Juice.

IV, 1, '11. Pancreatic juice, 1 cc.

Intestinal juice, 0.005 cc.
Sodium nucleinate solution, 10 per cent, 3 cc.

Control (1): Pancreatic juice, 1 cc.
Intestinal juice, 1 drop. (See cytidin experiment).
Salt solution, 3 cc.

Control (2): Intestinal juice, 0.005 cc.
Sodium nucleinate, 10 per cent, 3.5 cc.
Salt solution, 0.5 cc.

Control (3): Sodium nucleinate, 10 per cent. (See gastric juice experiment).

	10 min.	24 hrs.	72 hrs.	144 hrs.	168 hrs.
Experiment:	+4.32	+3.70	+3.45	+3.48	+3.48
Control (2):	+4.93	+4.85	+4.60	+4.50	+4.50

Free phosphoric acid, Fehling's solution, glacial acetic acid tests as in the precedent experiment.

EXPERIMENTS WITH INTESTINAL JUICE.

Experiment I.

III, 28, '11. Intestinal juice, 0.1 cc.
Sodium nucleinate, 6 per cent, 3.9 cc.

Control (1): Intestinal juice and (See guanylic acid experiment).
Salt solution.

Control (2): Sodium nucleinate, 6 per cent. (See experiment with pancreatic juice).

10 min.	24 hrs.	48 hrs.	96 hrs.	144 hrs.	200 hrs.
+2.75	+1.44	+1.15	+0.80	+0.60	+0.60

Free phosphoric acid present. Did not reduce Fehling's solution. Glacial acetic acid gave no precipitate.

Experiment II.

IV, 1, '11. Intestinal juice, 0.5 cc.
Sodium nucleinate solution, 10 per cent, 3.5 cc.

Control (1): Intestinal juice, and
Salt solution. (See guanylic acid experiment).

Control (2): Sodium nucleinate, 10 per cent. (See experiment with gastric juice).

10 min.	24 hrs.	72 hrs.	144 hrs.	168 hrs.
+4.70	+1.88	+1.40	+0.90	+0.83

Free phosphoric acid, Fehling's solution, glacial acetic acid tests as in the precedent experiment.

THYMUS NUCLEIC ACID EXPERIMENTS.

In physiological NaCl solution. Reading at the room temperature.

EXPERIMENT WITH NEUTRAL GASTRIC JUICE.

III, 28, '11. Gastric juice, 1 cc.
Sodium nucleinate, 5 per cent, 3 cc.

Control (1): Gastric juice, and
Salt solution. (See guanylic acid experiment.)

Control (2): Sodium nucleinate, 5 per cent.

	10 min.	24 hrs.	72 hrs.	144 hrs.
Experiment:	+1.08	+1.06	+1.05	+1.06
Control (2):	+1.45	+1.45	+1.44	+1.44

Hydrochloric acid gave a heavy precipitate.

Experiment with Unactivated Pancreatic Juice.

IV, 7, '11. Pancreatic juice, 1 cc.
Sodium nucleinate, 4.5 per cent, 3 cc.

Control (1): Pancreatic juice, and
Salt solution. (See guanylic acid experiment.)

Control (2): Sodium nucleinate solution. (See experiment with gastric juice.)

10 min.	24 hrs.	72 hrs.	120 hrs.
+0.98	+0.98	+1.00	+1.00

Hydrochloric acid gave a heavy precipitate.

Experiment with Activated Pancreatic Juice.

IV, 7, '11. Pancreatic juice, 1 cc.
Intestinal juice, 0.1 cc.
Sodium nucleinate, 4.5 per cent, 3 cc.

Control (1): Pancreatic juice, intestinal juice, and
Salt solution. (See cytidin experiment.)

Control (2): Sodium nucleinate solution. (See experiment with gastric juice.)

10 min.	24 hrs.	72 hrs.	144 hrs.
+0.98	+1.00	+1.02	+1.02

Hydrochloric acid gave a heavy precipitate.

Experiment with Intestinal Juice.

IV, 5, '11. Intestinal juice, 0.5 cc.
Sodium nucleinate, 4.5 per cent, 3.5 cc.

Control (1): Intestinal juice, and
Salt solution. (See guanylic acid experiment.)

Control (2): Sodium nucleinate solution. (See experiment with gastric juice).

10 min.	24 hrs.	72 hrs.	96 hrs.	144 hrs.	200 hrs.
+1.18	+1.17	+1.06	+0.90	+0.80	+0.70

Hydrochloric acid gave no precipitate. Free phosphoric acid present. Did not reduce Fehling's solution.

ON NUCLEASES.

SECOND PAPER.

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(Received for publication, April 28, 1911.)

In a previous communication on the same subject[1] the present writers reached the conclusion that the plasma of most of the organs of the dog contained enzymes which were capable of hydrolyzing nucleotides into ribose and a purin base. Such an enzyme was absent in the pancreatic gland. The pancreatic gland, however, possessed an enzyme which was capable of bringing about the detachment of phosphoric acid from the molecule of guanylic acid. The other organs contained enzymes capable of disintegrating inosinic and guanylic acids into their simple components. At the time of that investigation the impression was gained that the complete dissolution of the molecules was simultaneous without the formation of any intermediary complexes. The organs also contained enzymes which were capable of bringing about the cleavage of complex nucleic acids, such as the yeast nucleic acid. However, the extent of the change brought about in the molecule of these acids could not be ascertained with certainty. The organs apparently lacked enzymes capable of hydrolyzing pyrimidin complexes.

The view regarding the character of the alteration of guanylic acid, under the influence of pancreatic extract, was not considered conclusive. The character of the alterations produced by the plasma of various tissues on the thymus and on the yeast nucleic acids was also insufficiently explained. The cause for this lack of definite information lay partly in the complexity of the chemical structure of these acids, and also in the fact that at the time of that investigation the pyrimidin nucleotides had not yet been obtained, and therefore, the action of the tissue extracts on those complexes could not be ascertained.

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[1] Levene and Medigreceanu: This *Journal*, ix, p. 65, 1911.

Simultaneously with our first communication there appeared two publications by Walter Jones.[1] Regarding the action of the pancreatic gland on guanylic acid the views of this writer harmonize with the view expressed in our first publication, and previously indicated by Levene and Jacobs. The same writer has proven also that in course of nuclein fermentation the purin bases may undergo desamidation without becoming detached from their sugar complex.[2]

The present work was planned to fill the gaps left open in the previous investigation. Additional experiments were performed on guanylic acid. Further, observations were made on cytidin and uridin nucleotides, and new experiments were made on yeast and thymus nucleic acids.[3]

Also in the present work the changes in the optical rotation of the nucleic acid solutions served as a basis for the analysis of the character of the alterations brought about by the action of the enzymes. However, it was found advantageous to supplement the optical measurements by chemical tests. They consisted in an analysis for free phosphoric acid and for free reducing sugar. The organs of the dog exclusively were employed in all present experiments, as well as in those reported in the first communication.

The optical measurements were performed in the same manner as described in the first paper of this series.

The presence of free phosphoric acid was ascertained in the following manner. The solution was cooled in a freezing mixture and treated with ammonium molybdate solution as long as a precipitate formed. This amorphous, flocculent, white precipitate consisted of compounds of nucleic acid or nucleotides. To the filtrate ammonia water was added, the solution again cooled in a freezing mixture, and acidulated by means of nitric acid. In the presence of free phosphoric acid the typical yellow precipitate formed.

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[1] Walter Jones: This *Journal*, ix, pp. 129, 169, 1911.

[2] There has also appeared a communication by Juschtschenko on Nucleases, but the work has no bearing on our problem; *Biochem. Zeitschr.*, xxxi, 1911.

[3] The present investigation was nearly completed at the time of the publication of the articles by Professor Jones.

The test for free sugar was made by means of Fehling's solution. In the presence of purins and free sugar the white precipitate of the cuprous salt of purins formed.

In all experiments control tests were performed on the enzyme solutions, and on the solutions of the nucleic acids or of the nucleotides.

The results of the present investigation lead to the following conclusions.

Guanylic Acid. In the previous communication it was recorded that in the experiments with guanylic acids there was encountered frequently a great difficulty in obtaining accurate measurements of the optical rotation of the solution, for the reason that soon after the addition of the enzyme a turbidity appeared in the solution. On continued standing the turbidity developed into a crystalline precipitate. The precipitate consisted of free guanosin. This was established on the ground of the character of the crystalline appearance of the precipitate, and on the fact that a solution of the precipitate in dilute alkali possessed a higher rotatory power than the original solution of guanylic acid.

Through the action of the plasma of the pancreatic gland the digestion did not proceed beyond the stage of guanosin formation. The products of the action of the enzymes of that gland were phosphoric acid and guanosin.

The earlier finding by Levene and Jacobs[1] of free guanosin in the pancreatic gland is readily interpreted on the basis of the present experiments.

Under the influence of the extract of the intestinal mucosa, the following substances were formed: guanosin in form of a crystalline deposit, phosphoric acid, and free ribose. The presence of the latter was demonstrated by means of Fehling's solution. Guanosin was identified by its rotatory power and by the character of the crystalline appearance of the precipitate.

On the basis of these observations one is justified in assuming that the first phase in the cleavage of guanylic acid by means of the extract of the intestinal mucosa consists in the detachment of phosphoric acid from guanosin and the second phase consists in the hydrolysis of the nucleoside into sugar and purin base.

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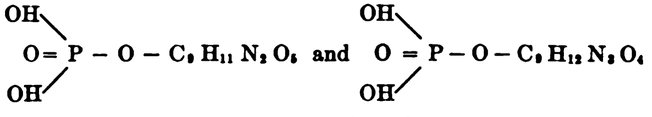
[1] Levene and Jacobs: *Biochem. Zeitschr.*, xxviii, p. 127, 1910.

Pyrimidin nucleotides. An equimolecular mixture of two nucleotides obtained from the yeast nucleic acid was employed in the present experiments. Their composition is expressed by the following formulas:



$$(OH)_2O{=}P-O-C_9H_{11}N_2O_6 \quad \text{and} \quad (OH)_2O{=}P-O-C_9H_{12}N_3O_4$$

It was noted by Levene and Jacobs that these nucleotides possessed a greater resistance against the influence of chemical hydrolytic agents than the purin nucleotides. The present experiments revealed an analogous behaviour of these complexes towards the action of nucleolytic enzymes.

Only the extract of the intestinal mucosa and the plasma of the kidney manifested a hydrolytic action on the pyrimidin nucleotides, and this was limited to a detachment of the phosphoric acid; the organic complex remaining undisturbed.

Owing to the comparatively low rotatory power of the solutions and to the slow velocity of the reaction the changes could not be detected by the optical method, and were demonstrated only by the appearance of free phosphoric acid in the solutions.

In none of the experiments did the solutions acquire the power to reduce Fehling's solution.

Yeast Nucleic Acid. This substance is composed chemically of at least four nucleotides, two of them containing purin-ribosides, and two of them containing pyrimidin complexes. The intact substance is insoluble in water, and is precipitated from the aqueous solution of its soluble salts by means of an excess of glacial acetic acid. On the other hand all known mono-nucleotides are soluble in water.

Under the influence of all extracts or plasma of the organs a solution of the sodium salt of this acid manifested the following alterations: first, a marked fall in the optical rotatory power; second, a change in its solubility, so that it no longer could be completely precipitated from the solution by means of glacial acetic acid. On addition of this reagent, often there appeared only a slight opalescence, in the experiments with some organs a turbidity, or only a slight precipitate, while in the control always a heavy precipitate was formed. In the previous communication the results of the analysis of the optical changes of the yeast nucleic acid were compared with those of its simpler fragments, if treated in the same manner. The conclusion was reached that even if the nucleic acid were to undergo disintegration into phosphoric acid, purin bases, d-ribose, cytidin and uridin, the solution of the split products would remain dextrorotatory, as was the original solution of the sodium salt of the nucleic acid.

In the present experiments evidence was secured to the effect that through the action of the intestinal mucosa on yeast nucleic acid, phosphoric acid, d-ribose, purin bases, and other complexes are formed. However, it was not possible to demonstrate that the acid underwent the same degree of cleavage under the influence of the enzymes contained in the plasma or extracts of other organs. It is possible that in the experiments in which the alterations were limited to those in the optical rotation and solubility, the cleavage did not proceed beyond the formation of mono-nucleotides.

Thymus Nucleic Acid similarly to the yeast nucleic acid belongs to the group of complex acids. There exist, however, decided differences in the chemical properties of the two substances. From the experiments, as yet unpublished, of Levene and Jacobs it has become evident that the phosphoric acid in the thymus nucleic acid cannot be detached with the same readiness as it is in the yeast. A similar difference is noticed between the two acids in their behavior towards enzymes.

Similarly to the yeast nucleic acid the thymus acid is insoluble, and is precipitated from a solution of its soluble salts by hydrochloric acid, though not by acetic.

The principal changes brought about in the thymus acid by the tissue enzymes consisted in a lowering of the optical rotation and an increase in its solubility, so that hydrochloric acid failed to cause its precipitation. Liberation of phosphoric acid was demonstrated only in the experiments with the extract of the intestinal mucosa. All of these changes were brought about at a decidedly lower intensity than in the yeast nucleic acid.

In none of the experiments could the formation of free purins or of free sugar be detected.

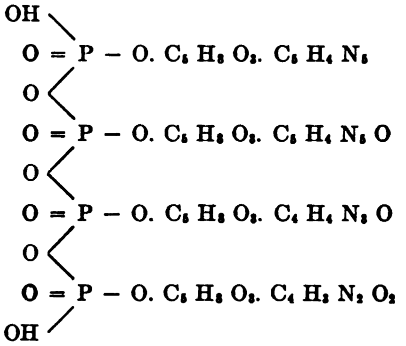
It is therefore possible, that under the conditions of the present experiments thymus nucleic acid is not hydrolyzed beyond the phase of the formation of nucleotides. Only by the action of the extract of the intestinal mucosa did the cleavage extend to the liberation of phosphoric acid, and therefore, to the formation of nucleosides. However, the intensity of the reaction is very low.

SUMMARY.

It has been known for some time, principally owing to the beautiful investigations of Walter Jones and his co-workers, that the final oxidation of the purin bases is the result of the graded action of several specific enzymes. Schittenhelm was led to the same conclusions. Very recently Jones demonstrated the existence of specific nucleoside-desamidases. On the ground of the present observations made on the action of tissue enzymes and of the enzymes of the gastro-intestinal tract on nucleic acids, the conclusion seems justified, that there exist in the organism several enzymes, which act harmoniously, leading to the disintegration of nucleic acids.

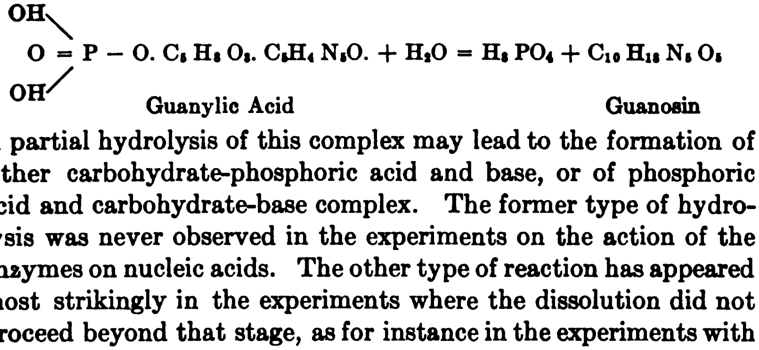
The function which hitherto has been ascribed to the "Nucleases" is in reality performed by at least three enzymes, or rather three groups of enzpmes, which have to be designated by special names. The part of each enzyme is to render the nucleic acid molecule susceptible to the action of one of the other enzymes, thus producing successive disintegration.

Yeast nucleic acid is a polynucleotide of the following structure:



It has not yet been definitely proven that the union of the individual nucleotides is brought about by the anhydride formation of the phosphoric acid, but it is certain that the four nucleotides are condensed in one molecule. In the process of disintegration these nucleotides are again detached. The plasma of some organs contain only the enzymes that are capable of bringing about this change but none of the other changes. Jones[1] has shown recently that the purin bases may be desamidized before the nucleoside is cleaved into its final components. Thus, some organs may contain only certain nucleases and at the same time certain desamidases. It may be found convenient to designate as *Nucleinases* the enzymes causing the dissolution of the nucleic acid molecule into nucleotides. As yet it is not certain whether the thymus nucleic acid and the yeast acid are cleaved into nucleotides by the same enzyme. *Nucleinase* is present practically in all organs, and in the pancreatic juice. It is absent in the gastric juice.

Nucleotides are composed of phosphoric acid and organic complexes formed through condensation of a carbohydrate and a base, and may be expressed in the following manner:



$$(OH)_2O = P - O.\,C_5H_8O_3.\,C_5H_4N_5O. + H_2O = H_3PO_4 + C_{10}H_{13}N_5O_5$$

Guanylic Acid Guanosin

A partial hydrolysis of this complex may lead to the formation of either carbohydrate-phosphoric acid and base, or of phosphoric acid and carbohydrate-base complex. The former type of hydrolysis was never observed in the experiments on the action of the enzymes on nucleic acids. The other type of reaction has appeared most strikingly in the experiments where the dissolution did not proceed beyond that stage, as for instance in the experiments with pancreas plasma and with the intestinal juice of the dog. The enzymes performing this cleavage may be referred to as *Nucleotidases*. They are present in the plasma of all organs, and in the intestinal juice; they are absent from the gastric and pancreatic juices.

Also in regard to *nucleotidases* the possibility is not excluded that there exists more than one enzyme of the same group, and this for the following reasons. From the work of Levene and Jacobs it has become apparent that the union of the phosphoric acid with the organic complex is of a different stability in different nucleotides. It is least stable in the guanylic acid and in the purin nucleotides of the yeast nucleic acid. It is more resistant in the pyrimidin nucleotides of the yeast nucleic acid and in the inosinic acid, and it is most resistant in the thymus nucleic acid. In accordance with this gradation in the stability of the nucleotides towards chemical agencies, there is noted a very decided distinction in their behavior towards enzymes. Thus, guanylic acid is hydrolyzed readily into phosphoric acid and guanosin by the plasma of the pancreatic gland, whereas there could not be ascertained the occurrence of the analogous cleavage of inosinic acid, of pyrimidin nucleotides, nor of the complex nucleic acids, through the action of the same plasma. On the other hand the cleavage takes place in all nucleotides under the action of the extract of the intestinal mucosa. This may give some foundation for the assumption of the existence of more than one nucleotidase.

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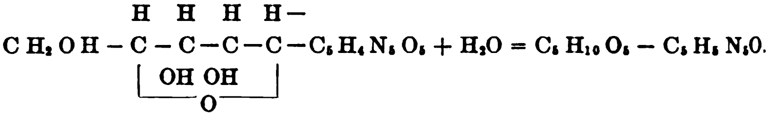
[1] W. Jones: This *Journal*, ix, p. 169, 1911.

Nucleosides are purin ribosides of the following structure:



$$CH_2OH-\underset{\text{OH}}{\overset{H}{C}}-\underset{\text{OH}}{\overset{H}{C}}-\overset{H}{C}-\overset{H-}{C}-C_5H_4N_5O_5+H_2O=C_5H_{10}O_5-C_5H_5N_5O.$$

Under the influence of mineral acids nucleosides are readily hydrolyzed into their components. The same cleavage can be brought about by the action of enzymes present in the plasma of most of the organs tested in that direction. This enzyme is absent in the plasma of the pancreatic gland, and in gastric, pancreatic and intestinal juices. Since this reaction is brought about by an enzyme different from the other nucleolytic enzymes, it may be proper to refer to it under the name of *Nucleosidase.*

Pyrimidin Complexes. Comparatively little information is obtained regarding the mechanism through which these substances undergo disintegration in the animal organism. The only evidence of the possible existence of enzymes bringing about cleavage of these complexes may be found in the older observation of Levene, that in course of prolonged autolysis of organs, free pyrimidins are formed.

GUANYLIC ACID EXPERIMENTS.

In physiological NaCl solution.

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA.

I, 17, '11. Enzyme solution, 1 cc.
Sodium guanylate, 6 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Physiological salt solution, 3 cc.

	10 min.	4 hrs.	24 hrs.	48 hrs.	72 hrs.
Experiment:	−0.43	−0.34	−0.29	−0.25	−0.23
Control:	0.02	−0.02	−0.02	−0.02	−0.02

Free phosphoric acid: present. Reduced Fehling's solution.

EXPERIMENT WITH PANCREAS PLASMA.

IV, 7, '11. Enzyme solution, 1 cc.
Sodium guanylate, 3 per cent, 3 cc.
(Reaction slightly alkaline)

Control: Enzyme solution, 1 cc.
Physiological salt solution, 3 cc.

	10 min.	2 hrs.	48 hrs.	96 hrs.
Experiment:	−0.30	Cloudy	−0.20	−0.20
Control:	+0.08	+0.08	+0.08	+0.08

A precipitate was formed consisting of characteristic guanosin crystals. Free phosphoric acid: present. Did not reduce Fehling's solution.

A 2.5 per cent solution of the air-dry precipitate in 10 per cent NaOH showed a rotation of −0.72 in a 5 cm. long observation tube. With the original guanylic acid under the same conditions the rotation was only −0.40.

PYRIMIDIN NUCLEOTIDE EXPERIMENTS.

In physiological NaCl solution.

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA.

IV, 15, '11. Enzyme solution, 1 cc.
Nucleotide solution, 3 cc.

Control: Enzyme solution, 1 cc.
Salt solution, 3 cc.

	30 min.	24 hrs.	48 hrs.	92 hrs.
Experiment:	+0.20	Cloudy	+0.12	+0.12
Control:	0.00	0.00	0.00	0.00

Free phosphoric acid: present. Did not reduce Fehling's solution.

EXPERIMENT WITH PANCREAS PLASMA.

IV, 15, '11. Enzyme solution, 1 cc.
Nucleotide solution, 3 cc.

Control: Enzyme solution, 1 cc.
Salt Solution, 3 cc.

	10 min.	24 hrs.	144 hrs.
Experiment:	+0.20	+0.18	+0.18
Control:	+0.08	+0.07	+0.07

Free phosphoric acid: absent.

EXPERIMENT WITH LIVER PLASMA.

IV, 15, '11. Enzyme solution, 1 cc.
Nucleotide solution, 3 cc.

Control: Enzyme solution, 1 cc.
Salt solution, 3 cc.

	10 min.	24 hrs.	96 hrs.	144 hrs.
Experiment:	+0.20	+0.18	+0.16	+0.16
Control:	+0.30	+0.30	+0.30	+0.30

Free phosphoric acid: doubtful.

EXPERIMENT WITH KIDNEY PLASMA.

IV, 15, '11. Enzyme solution, 1 cc.
Nucleotide solution, 3 cc.

Control: Enzyme solution, 1 cc.
Salt solution, 3 cc.

	10 min.	24 hrs.	96 hrs.
Experiment:	+0.20	Cloudy	+0.10
Control:	−0.04	−0.04	−0.04

Free phosphoric acid: present. Did not reduce Fehling's solution.

EXPERIMENT WITH HEART MUSCLE PLASMA.

IV, 15, '11. Enzyme solution, 1 cc.
Nucleotide solution, 3 cc.

Control: Enzyme solution, 1 cc.
Salt solution, 3 cc.

	10 min.	24 hrs.	96 hrs.	144 hrs.
Experiment:	+0.20	cloudy	+0.18	+0.10
Control:	−0.02	cloudy	−0.02	−0.02

Free phosphoric acid: absent. Did not reduce Fehling's solution.

EXPERIMENT WITH BLOOD SERUM.

IV, 15, '11. Blood serum, 1 cc.
Nucleotide solution, 3 cc.

Control: Blood serum, 1 cc.
Salt solution, 3 cc.

	10 min.	24 hrs.	144 hrs.
Experiment:	+0.19	+0.19	+0.18
Control:	−0.32	−0.32	−0.32

Free phosphoric acid: absent.

EXPERIMENT WITH HEMOLYZED BLOOD.

(With ether and distilled water)

IV, 15, '11. Blood, 1 cc.
Nucleotide solution, 3 cc.

Control: Blood, 1 cc.
Salt solution, 3 cc.

	10 min.	24 hrs.	144 hrs.
Experiment:	+0.20	+0.21	+0.20
Control:	0.00	cloudy	0.00

Free phosphoric acid: absent.

YEAST NUCLEIC ACID EXPERIMENTS.

In physiological NaCl solution. Readings at room temperature.

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA.

IV, 8, '11. Enzyme solution, 0.5 cc.
Sodium nucleinate, 6 per cent, 3 cc.
Salt solution, 0.5 cc.

Control (1): Enzyme solution, 0.5 cc.
Salt solution, 3.5 cc.

Control (2): Sodium nucleinate, 6 per cent.

	10 min.	24 hrs.	48 hrs.	96 hrs.	192 hrs.
Experiment:	+2.38	+1.30	+1.00	+0.60	+0.60
Control (1):	0.00	0.00	0.00	0.00	0.00
Control (2):	+3.35	+3.25	+3.25	+3.12	+3.12

Free phosphoric acid: present. Reduced Fehling's solution. Glacial acetic acid gave no precipitate.

EXPERIMENT WITH PANCREAS PLASMA.

IV, 18, '11. Enzyme solution, 1 cc.
Sodium nucleinate, 6 per cent, 3 cc.

Control (1): Enzyme solution, 1 cc.
Salt solution, 3 cc.

Control (2): Sodium nucleinate, 6 per cent, see preceding experiment.

	10 min.	24 hrs.	48 hrs.	96 hrs.	192 hrs.
Experiment:	+2.45	+2.10	+1.90	+1.60	+1.40
Control (1):	+0.08	+0.08	+0.08	+0.08	+0.08

Glacial acetic acid gave a slight precipitate. Free phosphoric acid: absent.

Experiment with Liver Plasma.

IV, 8, '11. Enzyme solution, 1 cc.
Sodium nucleinate, 6 per cent, 3 cc.

Control (1): Enzyme solution, 1 cc.
Salt solution, 3 cc.

Control (2): Sodium nucleinate, 6 per cent, see experiment with extract of intestinal mucosa.

	10 min.	24 hrs.	48 hrs.	96 hrs.	192 hrs.
Experiment:	+2.45	+1.38	+1.25	+1.40	+1.35
Control (1):	+0.30	+0.30	+0.30	+0.30	+0.30

Free phosphoric acid: absent. Glacial acetic acid gave a slight precipitate.

Experiment with Kidney Plasma.

IV, 8, '11. Enzyme solution, 1 cc.
Sodium nucleinate, 6 per cent, 3 cc.

Control (1): Enzyme solution, 1 cc.
Salt solution, 3 cc.

Control (2): Sodium nucleinate, see experiment with extract of intestinal mucosa.

	10 min.	24 hrs.	48 hrs.	96 hrs.	192 hrs.
Experiment:	+2.40	cloudy	+1.45	1.25	+1.25
Control (1):	−0.06	cloudy	−0.05	−0.05	−0.05

Glacial acetic acid gave a slight precipitate. Free phosphoric acid: absent. Did not reduce Fehling's solution.

Experiment with Heart Muscle Plasma.

IV, 8, '11. Enzyme solution, 1 cc.
Sodium nucleinate, 6 per cent, 3 cc.

Control (1): Enzyme solution, 1 cc.
Salt solution, 3 cc.

Control (2): Sodium nucleinate, see experiment with extract of intestinal mucosa.

	10 min.	24 hrs.	48 hrs.	96 hrs.	192 hrs.
Experiment:	+2.42	+1.65	+1.52	+1.20	+0.98
Control (1):	−0.02	−0.02	−0.02	−0.02	−0.02

Glacial acetic acid gave a very slight precipitate. Free phosphoric acid: absent. Did not reduce Fehling's solution.

Experiment with Blood Serum.

IV, 8, '11. Blood serum, 1 cc.
Sodium nucleinate, 6 per cent, 3 cc.

Control (1): Blood serum, 1 cc.
Salt solution, 3 cc.

Control (2): Sodium nucleinate, see experiment with extract of intestinal mucosa.

	10 min.	24 hrs.	48 hrs.	96 hrs.	192 hrs.
Experiment:	+2.40	+1.47	+1.37	+1.17	+1.05
Control (1):	−0.32	−0.32	−0.32	0.32	−0.32

Free phosphoric acid: absent.

Experiment with Hemolyzed Blood.

(With ether and distilled water.)

IV, 8, '11. Blood, 1 cc.
Sodium nucleinate, 6 per cent, 3 cc.

Control (1): Blood, 1 cc.
Salt solution, 3 cc.

Control (2): Sodium nucleinate, see experiment with extract of intestinal mucosa.

	10 min.	24 hrs.	96 hrs.	192 hrs.
Experiment:	+2.35	+2.18	+2.20	+2.05
Control:	0.00	0.00	dark	dark

Free phosphoric acid: absent.

THYMUS NUCLEIC ACID EXPERIMENTS.

In physiological NaCl solution. Readings at room temperature.

Experiment with Extract of Intestinal Mucosa.

IV, 8, '11. Enzyme solution, 0.5 cc.
Sodium nucleinate, 5 per cent, 3.5 cc.

Control[1] (1) Enzyme solution, and
Salt solution, see the experiment with yeast nucleic acid.

Control[2] (2): Sodium nucleinate, 5 per cent.

	10 min.	24 hrs.	72 hrs.	144 hrs.
Experiment:	+1.20	+0.85	+0.77	+0.70
Control (2):	+1.36	+1.35	+1.34	+1.35

Free phosphoric acid: present. Did not reduce Fehling's solution. Hydrochloric acid gave a precipitate.

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[1] For the controls—enzyme and salt solution—see the corresponding experiments with yeast nucleic acid.

[2] This control holds good for all the thymus nucleic acid experiments here mentioned.

Experiment with Pancreas Plasma.

IV, 8, '11. Enzyme solution, 1 cc.
Sodium nucleinate, 5 per cent, 3 cc.

10 min.	24 hrs.	48 hrs.	144 hrs.
+0.94	+0.27	+0.20	+0.20

Hydrochloric acid gave a very slight precipitate.

Experiment with Liver Plasma.

IV, 8, '11. Enzyme solution, 1 cc.
Sodium nucleinate, 5 per cent, 3 cc.

10 min.	24 hrs.	96 hrs.	144 hrs.
+1.00	+0.95	+0.95	+0.90

Hydrochloric acid gave a heavy precipitate.

Experiment with Kidney Plasma.

IV, 8, '11. Enzyme solution, 1 cc.
Sodium nucleinate, 5 per cent, 3 cc.

10 min.	24 hrs.	96 hrs.	192 hrs.
+1.00	cloudy	+0.60	+0.30

Hydrochloric acid gave a slight precipitate. Free phosphoric acid: doubtful. Did not reduce Fehling's solution.

Experiment with Heart Muscle Plasma.

IV, 8, '11. Enzyme solution, 1 cc.
Sodium nucleinate, 5 per cent, 3 cc.

10 min.	24 hrs.	192 hrs.
+1.00	+0.95	+0.94

Hydrochloric acid gave a heavy precipitate. Free phosphoric acid: absent. Did not reduce Fehling's solution.

Experiment with Blood Serum.

IV, 8, '11. Blood serum, 1 cc.
Sodium nucleinate, 5 per cent, 3 cc.

10 min.	24 hrs.	192 hrs.
+0.98	+0.95	+0.90

Hydrochloric acid gave a heavy precipitate. Free phosphoric acid: absent.

A CRITICAL STUDY OF THE PROCESS OF ACID EXCRETION.[1]

By LAWRENCE J. HENDERSON.

(From the Laboratory of Biological Chemistry of the Harvard Medical School.)

(Received for publication, May 9, 1911.)

The right working of physiological processes depends upon accurate adjustment and preservation of physico-chemical conditions within the organism. Three such conditions, temperature, molecular concentration, and neutrality are now known to be nicely adjusted and maintained; adjusted by processes going on in the body, maintained by exchanges with the environment. This paper is concerned with the physiological processes whereby the normal reaction of the body fluids, through regulation of the excretion of acid and basic material, is permanently maintained.

Throughout the animal body, while life exists, there occurs a regular formation of acid substances, excretory products of metabolism. As they form, these various matters, carbonic acid, sulphuric acid, and phosphoric acid in the main, immediately combine, but only partially, according to their several avidities, with the basic constituents of protoplasm and blood. In pathological conditions great quantities of acetoacetic acid and β-oxybutyric acid may be produced and claim their share of base. Thus, through resulting changes in equilibria between bases and acids, normal metabolism steadily operates to lower the unvarying alkaline reaction[2] of the body. This tendency is held sharply in check by special protective mechanisms, acting coördinately, in coöperation and regular succession.

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[1] The substance of this paper was presented at the Eighth International Physiological Congress, Vienna, September, 1910.

[2] Or neutrality. The terms may be used interchangeably for an alkalinity which is so slight. The reaction at 18° is actually that of a sodium hydrate solution of strength 0.0000003N, containing 0.000012 gm. NaOH per liter.

The chemical reactions whereby such material is first neutralized, the chemical substances which aid in neutralization, the shares of more important substances in the process, and their efficiency, the changes in chemical equilibria, including resulting changes in hydrogen and hydroxyl ion concentrations, all, so far as they concern true solution, are known with a fair approach to certainty.[1] Principally this work of neutralization is done by salts of phosphoric and carbonic acids, with aid from the amphoteric proteins.[2] In simplified form the process may be represented by the two reactions, $M_2HPO_4 + HA = MA + MH_2PO_4$; $MHCO_3 + HA = MA + H_2CO_3$, where M stands for any basic radical, A for any acid radical. Other less important simultaneous reactions are of the same type, except perhaps the union of the weak acids with basic proteins like globine, and the union of bases with more acid proteins. Through the remarkable circumstance that phosphates and carbonates possess, among all known chemical substances, the highest power to preserve neutrality in solution,[3] this function is so well performed that the alkaline reaction of the body scarcely varies, even when the load upon the mechanism is heavy.

But, however efficient such an arrangement may be, it is of its very nature only the first stage in the process of the excretion of acid, and wholly dependent upon constant support by the kidney, and of course upon a supply of alkali in the food. Regularly, as r form, the acid bodies must be afforded alkali by blood and oplasm, for every mole of carbonic acid about 0.93 mole of li, for every mole of phosphoric acid about 1.89 moles of alkali, for every mole of sulphuric acid 2.00 moles of alkali, in accord- with chemical laws and the normal reaction of the body.[1] rly therefore this neutralization must rest upon physiological esses which serve to reëstablish the original conditions, for if great amount of alkali were discharged from the body with acid excretory substances, the organism would lose its protec- and acidity would speedily ensue throughout the system. s an imperative necessity arises for the retention of a part of alkali which serves as a carrier in the process of removing acid n the body. Of course the necessary magnitude of such alkali ntion by the kidney varies with the net amount of alkali in- ed and with the acid formation of the body.

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[1] L. J. Henderson: The Theory of Neutrality Regulation in the Animal Organism, *American Journal of Physiology*, xxi, p. 427, 1908; and *Ergebnisse der Physiologie*, Das Gleichgewicht zwischen Basen und Säuren im tierischen Organismus, viii, p. 254, 1909. See also Robertson: this *Journal*, vii, p. 351, 1910.

[2] L. J. Henderson: On the Neutrality Equilibrium in Blood and Protoplasm, this *Journal*, vii, 29, 1909, and T. Brailsford Robertson: *loc. cit.* These two papers clearly show that the serum proteins are of considerable secondary importance in the process, through variation in the amount of alkali which they bind. Probably the major part of the protein content of the body is similar to these substances in power to combine with alkali in solution. What may be the case with undissolved proteins, through heterogeneous equilibrium and adsorption phenomena, we cannot say. In this connection one naturally thinks of the acid nucleoproteins.

[3] L. J. Henderson: *American Journal of Physiology*, xxi, p. 173, 1908.

ortunately carbonic acid, because of its very weak acidity, readily be liberated from chemical combination. Its easy version into gaseous carbon dioxide then permits it to leave the y in the free state, though not without the intervention of a arkable chemical mechanism[2] whereby acid is provided to bine in blood plasma in the lung capillaries with the alkali ch in transport has been combined with the carbonic acid. s no difficulty exists with the excretion of this chief acid pro- t of metabolism, and the store of alkali which serves for its

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n the general equation for such equilibria $(\overset{+}{H}) = \frac{k}{\gamma} \times \frac{HA}{NaA}$, $k_{H_2\bar{P}O_4}$ $\times 10^{-7}$, $k_{H_2CO_3}$ is 3×10^{-7}, and γ (the degree of ionisation of the salt) out 0.8. The hydrogen ion concentration of blood is 0.3×10^{-7}. It ws that at 18°

$$\times 10^{-7} = \frac{2 \times 10^{-7}}{0.8} \times \frac{NaH_2PO_4}{Na_2HPO_4} \text{ and } 0.3 \times 10^{-7} = \frac{3 \times 10^{-7}}{0.8} \times \frac{H_2CO_3}{NaHCO_3}$$

nce

$$\frac{NaH_2PO_4}{Na_2HPO_4} = \frac{1}{8.3} \text{ and } \frac{H_2CO_3}{NaHCO_3} = \frac{1}{12.5}$$

t is to say that of the phosphates of protoplasm and blood 89 per cent of the type M_2HPO_4 and 11 per cent of the type MH_2PO_4, while of the l carbonic acid 93 per cent is in the form of bicarbonate and 7 per cent . For a complete discussion of this matter see Henderson: *Ergebnisse Physiologie*, viii, pp. 263-271, 1909.

L. J. Henderson: this *Journal*, vii, p. 34, 1909.

temporary fixation, about 1.5 grams in the blood at any moment, or about 400 grams daily, is wholly saved.

With phosphates and sulphates, however, the case is different, and these substances, as they exist in the urine, still bind alkali. In fact even in the acid urine such stronger acids are combined with much base, phosphoric acid with at least nearly one equivalent (NaH_2PO_4), sulphuric acid with almost exactly two (Na_2SO_4). Much less alkali than this would necessitate intense acidity of reaction.

In several ways, however, such drain of alkali through the kidney is checked and, by variation in the urinary balance of base and acid, regulated. Thus the organ is enabled very nicely to preserve within the body that balance between basic and acid substances which is essential to life itself. The logical analysis of these processes, constituting the physiological arrangement for preserving and constantly restoring the primary mechanism of neutrality regulation, is the subject of the present paper.[1]

THE EXCRETION OF ACID.

Normal urine is a little more acid than the body as a whole; on the average its concentration in ionized hydrogen is perhaps twenty times that of blood,[2] but the urinary reaction is relatively very variable. Though the absolute magnitude of this difference in true acidity between the two fluids is very slight, the urine contains, as a result of it, and of the nature of some of its constituents, relatively not a little more acid and less base than the blood. In this manner a considerable portion of the bases which in the circulating blood are in chemical union with the acids of the urine remain in the body, retained by the kidney, after the excretion of the acids. These bases are now once more in union with the substances from which they were originally split off by acid as it formed in the tissue. In other words as acid is taken out of the blood in the kidney the reactions for the neutralization of acid are in effect there reversed.

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[1] The following discussion is based upon experimental studies which have been carried on in this laboratory. See Fitz, Alsberg, and Henderson: *American Journal of Physiology*, xviii, p. 113, 1907; Henderson and Adler: *Proceedings Am. Soc. Biol. Chemists*, 1908, p. xxxviii, and Henderson: *Biochemische Zeitschrift*, xxiv, p. 40, 1910. In some respects it is an extension of a paper by Henderson and Spiro: *Biochemische Zeitschrift*, xv, p. 105, 1908.

[2] The average values at 18° are 5×10^{-7} and 0.3×10^{-7} hydrogen ion concentration respectively.

Another and quite different factor in the regulatory process is the urinary ammonia, which of course has long been known to serve the same purpose of saving alkali for the body. The amount of alkali thus saved is precisely equivalent to the total amount of this urinary ammonia, because the substitution of ammonia in place of other bases is a simple chemical process. Furthermore ammonia is a product of metabolism which, formed from nearly neutral substances, is ordinarily destined to produce a neutral substance, urea. Such ammonia then represents a clear gain of so much alkali for the body.

These two factors, the physico-chemical separation of acid, and ammonia excretion, are separate, they are chemically independent, and they are strictly additive. Under ordinary circumstances the two are probably of about the same order of magnitude. The one factor is the result of a relatively more effective removal of acid than of base from the blood, as it flows through the kidney, a process which may perhaps be due in part to that tendency, observed by Maly,[1] of the acid constituents to diffuse and to dialyze most rapidly from such mixtures as are found dissolved in blood. Purely physical separation in a laboratory experiment like Maly's depends upon the greater mobility of ionized hydrogen and of more complex substances which contain hydrogen ($H_2\overset{-}{P}O_4$), than of corresponding basic substances. Undoubtedly however the physiological process is too well regulated to be without effective physiological control, and the simple physical phenomenon seems to be constantly and widely modified to meet the needs of the occasion. At best it constitutes an underlying constant tendency which may be wholly obscured by independently varying physiological processes. The second factor, excretion of ammonia, is in some respects of a more highly specialized sort. This phenomenon seems to be a selective excretory activity of the kidney whereby in the urine ammonia, so far as it is available, is almost quantitatively substituted for other bases of the blood. With the origin of this ammonia we are not here concerned.

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[1] Maly: *Liebig's Annalen*, clxxiii, p. 227, 1874; *Berichte der deutschen chemischen Gesellschaft*, ix, p. 164, 1876; *Zeitschrift für physiologische Chemie*, i, p. 174, 1877; *Pflüger's Archiv*, xxii, p. 111.

The amount of alkali retained through the operation of each of these processes is highly variable, in accordance with the immediate need of the body. Through such variations the physiological equilibrium between base and acid is maintained constant, during periods of varying intake and formation of acid, just as the constant molecular concentration (osmotic pressure) of body fluids is preserved by variations in the molecular concentration of the urine. Likewise similar in its logical aspects is the regulation of the third great constant condition, body temperature, through variations in the output of heat.

From the point of view of total metabolism the end of the two processes is to remove the base which, entering the body with the food, passes through unchanged (sodium, potassium, calcium, magnesium, etc.), plus that which is formed from neutral substances in the body (chiefly ammonia), together with the acid which passes through the body unchanged (chlorides, sulphates, phosphates, etc.) plus that which is formed in metabolism (sulphates, phosphates, oxybutyrates, and the like). Here this aspect of the matter calls for no special comment, for it is not unlike other phases of total metabolism.

The acid and alkali balance of renal activity needs further analysis, though from the standpoint of physiological economics one factor therein, the nature of the process whereby urinary ammonia does its work, is not in doubt. As we have seen, ammonia in the urine is equivalent to the bases in the blood[1] for which it is substituted. On the other hand the rôle of urinary acidity in preserving blood alkalinity is a complex matter, but from the point of view of physical chemistry its analysis presents no serious difficulty. Two principal considerations are involved in the definition of this process;—the magnitude of the excretion of acid, and the efficiency of the kidney as a separator of acid from the blood.

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[1] In the acid urine salts of ammonia are not appreciably hydrolysed. Therefore ammonia fully neutralizes its equivalent of strong acid.

THE MAGNITUDE OF THE EXCRETION OF ACID.

All methods of titration of urinary acidity are designed to measure excess of acid over basic substances in the urinary solution. In every case these methods possess in the use of an indicator one important qualification, a qualification which rigidly limits their precise quantitative significance.

Without exception such procedures consist in titrating to the reaction which chances to be a convenient "end-point" of a particular indicator. But such a point is in itself quite without necessary physiological significance, for there is in the nature of things no relation between any physiological constant or process and the reaction at which phenolphthalein, or litmus, or alizarine, or any other indicator changes color sharply. Each of these concentrations of ionized hydrogen is dependent in an indeterminate way upon the chemical constitution of the indicator, and upon nothing else. Any other relationship is purely accidental. Nor is there even in chemical theory that prescription of a particular indicator which may always be found in the case of the titration of any simple solution. This fact is amply attested by the difficulties which have been encountered in devising a suitable titration method to estimate the excess of acid over base in urine. Among the urinary constituents are some whose "neutralization" is incomplete at any possible end point. In solutions which are just pink with phenolphthalein ammonia is free in measurable amount, and proteins and amino-acids are combined with a considerable and uncertain quantity of base. Between such alkalinity and the acidity of the methyl orange end point the phosphates and many other substances are combined with varying amounts of base. Finally at a more acid reaction hippuric acid, lactic acid, and other substances are present partly as salts and partly free, while, on the other hand, the proteins and their derivatives are in part united with acid.

Much the sharpest of the common end points in urinary analysis is that of phenolphthalein, and therefore, in the estimation of excess of acid over base in urine, in studying total metabolism for example, results obtained with this indicator serve, through comparisons among themselves, a very useful purpose. Indeed disregarding ammonia, the one important urinary constituent which is a moderately weak base, and all the very weak basic substances of the solution, this end point does very nearly mark a series of completed chemical reactions. Yet it is hard to see how such results can be utilized for the study of the last step of intermediary metabolism, the regulatory activity of the kidney, if for no other reason because the blood too has a considerable "acidity" as measured with this indicator. Certainly such results measure even approximately neither the intensity of acidity in urine, nor the excess of acid there present as compared with blood.

The blood has an extraordinarily constant reaction, on the average 0.3×10^{-7} N or 0.4×10^{-7} N in ionized hydrogen. Fortunately, through utilization of this fact, the extent of the physiological process with which we are here concerned may be readily studied by titration. But suitable special means must be employed.

When alkali is added to the urine until the hydrogen ion concentration has become precisely equal to that of normal blood, the amount of alkali added must of course, disregarding the very slight effect of dilution, an effect which varies from constituent to constituent, be equal to that which the kidney has held back by physical means during the formation of the urine. Such an addition of alkali merely reverses that process which has been carried out in the kidney in order to save alkali for the body.

This quantity is therefore within the limits of accuracy of indicators the physiological acid excretion as above defined, in short the amount of acid in excess of the normal quantity of acid in blood of which the body has freed itself.[1] The difference between such a quantity and that measured by titration with phenolphthalein may be illustrated as follows. A hundredth molal solution of monosodium phosphate, when titrated with phenolphthalein, appears to be about 0.010 normal in acidity. The same solution when titrated to the reaction of the blood appears about 0.009 normal in acidity. A solution containing monosodium phosphate and disodium phosphate, the former 0.01 molal, the latter 0.05

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[1] The effect of differences in dilution cannot be more than 10 per cent in the absolute magnitude of the hydrogen ion concentration, which can cause no appreciable variation except in the case of the phosphates. But such slight variations, *e.g.*, $\overset{+}{(\mathrm{H})} = 3.0 \times 10^{-8}$ or $\overset{+}{(\mathrm{H})} = 3.3 \times 10^{-8}$, certainly occur in blood itself.

molal is, when titrated with phenolphthalein, also about 0.010 normal. When titrated to blood alkalinity however the latter solution is only about 0.003 normal. Evidently the results of the two titrations vary independently.

It is evident that the total concentration of acid radicals, free and combined, in urine is without significance for acid excretion. Likewise evident is the impossibility of assigning any meaning to the results of titration to an exactly neutral reaction, with its characteristic balance of the two salts of phosphoric acid in the proportion

$$NaH_2PO_4 : Na_2HPO_4 = 1 : 3$$

Finally, even if it were possible to titrate to a point at which all the bases and acids are exactly "neutralised," the present goal would be no nearer, because such a condition is far from that which occurs in the blood. Indeed the only means of reaching an understanding of urinary acidity, in its bearing upon the formation of urine from blood, is logically to distinguish between one moiety of acid radicals in the urine which stand for acid which in the blood was combined with the sum of all the bases now in the urine, and another moiety which is, as above stated, the true acid excretion. It matters not at all that this acid as it exists in urine, is, through readjustment of chemical equilibrium, largely combined with base.

With "neutral red" the reaction of the blood is rather sharply indicated by a reddish brown color which quickly becomes more reddish if acid be added, more brown if the alkalinity be increased. When using this indicator in urinary titration it is therefore possible to mark the physiological end point. The magnitude of acid excretion or, what amounts to the same thing, alkali retention, may therefore be measured as follows.[1] A flask of 250 cc. capacity is filled with a phosphate solution consisting of 7.4 parts disodium phosphate and 1.0 part monosodium phosphate. The dilution should be such that the concentration of total phosphoric acid is about 0.1 molal. This solution has the reaction of the blood.[2]

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[1] Henderson and Adler: *loc. cit.*; see also Adler and Blake: *Archives of Internal Medicine*, vii, p. 479, 1911.

[2]
$$0.33 \times 10^{-7} = \frac{2 \times 10^{-7}}{0.83} \times \frac{1.0}{7.4}$$

Twenty-five cc. of urine are now diluted with water until the volume is nearly 250 cc. A wise and important precaution is first to precipitate calcium with a small quantity of neutral potassium oxalate solution as suggested by Folin.[1] This should be done in spite of the fact that commonly even without such treatment no precipitate forms during the titration. The precipitate of calcium oxalate must be removed by filtration. The two solutions are now treated with equal quantities of neutral red solution, and the urine is titrated until the colors match. The dilution of the urine, designed to eliminate its natural color, serves also to produce a solution in which the degree of ionization is not unlike that in blood. Thereby the process becomes more accurate, for it is only through slight variations in degree of ionization that one fails accurately to reëstablish the equilibrium between acids and bases as originally present in the blood. Another advantage of dilution is to be found in the diminution of the very slight effect of the neutral salts upon the color of the indicator. However, in any case, the error introduced by these influences is really insignificant.[2] The results are much lower than those obtained with phenolphthalein, by the ordinary method of titration, but there is, as we have seen, no quantitative relationship between the data of the two methods.

The sole advantage of this procedure consists in titrating to blood alkalinity. This is however the only means of obtaining values of known significance concerning the physiological process by titration of urinary acidity.

The data of such titrations are to be calculated for the total volume of urine. The result of this calculation plus the total urinary ammonia, as above fully explained, measures the total excretion of acid, that is to say the total excess of acid above the normal amount present in the blood and there associated with the urinary bases, which has been removed by the kidney. It measures also the total retention of alkali, because an equivalent quantity of alkali has been separated from this acid in the kidney, and restored to the circulating blood.

In extreme acidosis, there may be an appreciable diminution in blood alkalinity. To such rare cases, the method is, without slight modification, inapplicable.

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[1] Folin: *American Journal of Physiology*, xiii, p. 178, 1905.

[2] The difficulties are merely those inherent in the use of indicators.

THE EFFICIENCY OF THE PROCESS.

When the amount of acid separated from the blood by the kidney has been measured, in the manner above described, one possesses all the information that titration can at present yield regarding this physiological process. None the less is the efficiency with which the kidney is doing its work unknown, for the amount of work done is no measure of the efficiency of the process. Evidently a given excess, in the physiological sense, of acid over base in urine may depend upon the elimination of a large amount of material of relatively low intensity of acidity or upon the elimination of a smaller amount of material of higher acidity, which therefore contains relatively little base. In other words the excess of acid above that present in blood may be associated with either a large or a small amount of saline material consisting of acids and bases in the proportion found in the blood. Thus the efficiency of the process varies with the relative and not with the absolute excess of acid over basic material in the urine, and it is in fact only to be estimated by a very different process from titration, the measurement of the actual acidity or hydrogen ion concentration.

Whenever a weak acid and its salt with a strong base like sodium are present together in solution the hydrogen ion concentration, that is to say the intensity of acidity, is proportional to the ratio between free acid and sodium salt. Moreover the factor of proportionality is very nearly equal to the ionization constant of the acid,[1] and thus an approximate general equation for such cases may be written $(\overset{+}{\mathrm{H}}) = k\,\frac{\mathrm{HA}}{\mathrm{NaA}}$

Here $(\overset{+}{\mathrm{H}})$ indicates the concentration of ionized hydrogen, k the ionization constant of the acid, HA and NaA the total molecular concentrations of acid and salt respectively. Such relations persist in dilute aqueous solutions, no matter what other substances may be there present. Accordingly when the ionization constant of an acid is known, and also the intensity of acidity of a solution in which it is present, it is a simple matter to calculate the relative amount of the acid which is free in solution, and the relative amount which is in combination with base. The ionization constants of the important urinary acids have now been determined.[1] These quantities are as follows.

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[1] Actually this quantity is equal to the ionization constant of the acid divided by the degree of dissociation of the salt.

Hippuric acid	0.00022
Acetoacetic acid	0.00015
Lactic acid	0.00013
β-Oxybutyric acid	0.00002
Uric acid	0.0000015
Carbonic acid	0.0000003
MH_2PO_4	0.0000002

From these data, with the help of the equations of the concentration law, the accompanying curves are constructed.

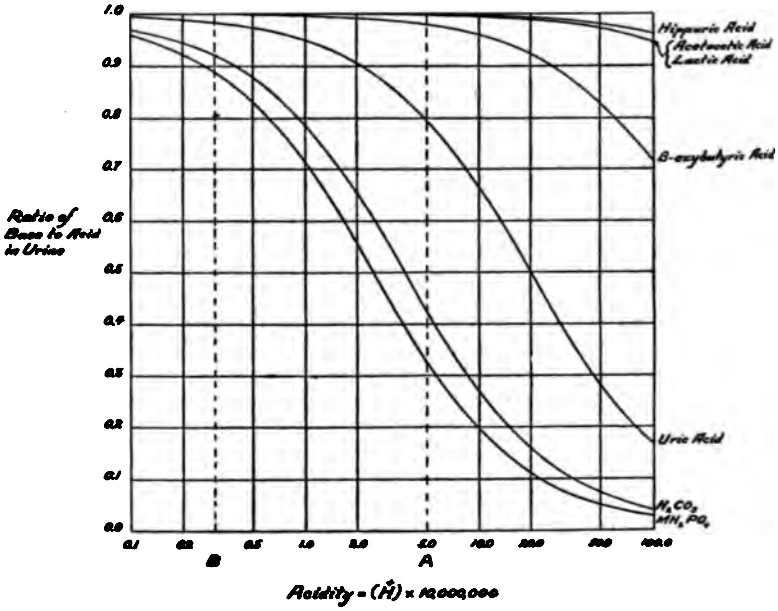

FIG. I. Reaction, expressed in terms of concentrations of ionized hydrogen, is plotted logarithmically on the axis of the abscissas. For convenience these concentrations are multiplied by 10,000,000. Ordinates represent the amount of base combined with one equivalent of acid. The lines *B* and *A* correspond to the reaction of the blood and to the mean reaction of the urine respectively. The several curves correspond to the urinary acids indicated.

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[1] For a discussion of this subject see Henderson and Spiro: *Biochemische Zeitschrift*, xv, p. 105, 1908.

These curves show the equilibria between acids and salts, in ch solutions as are found in urine. Evidently phosphoric acid, th on account of the magnitude of its avidity for base and on count of the relatively great amount present in urine, is that one the urinary acids which varies most in its ability chemically bind base as the acidity of the urine changes within physiologi- l limits. Thus chiefly through the presence of this substance the ine can carry out from the body varying amounts of acid in mbination with a given amount of base. Accordingly it fol- ws that phosphoric acid is primarily concerned, not only with the utralization of acid in the body, but also with the preservation the normal physiological mechanism of neutrality regulation means of renal activity. For this task it possesses no less than eal qualifications, as will be at once evident from a consideration the actual intensity of the urinary acidity.

Quite recently measurements of the normal urinary acidity have en made in this laboratory in sufficient number to define the dinary conditions of the equilibrium.[1] These studies were rried out with indicators in urines diluted to such a degree that e normal color was nearly annulled and the influence of neutral bstances upon the indicator minimized. A series of solutions of own reaction was prepared in the customary manner by mixing rying quantities of acid and salt. The following table shows e constitution and the appromixate reaction of these solutions:

No.	(NaH_2PO_4)	(Na_2HPO_4)	$(\overset{+}{H})$	Indicator
....	0.0010N	0.0060N	4×10^{-8}N	Neutral red
....	0.0010N	0.0023N	1×10^{-7}N	
	(CH_3COOH)	(CH_3COONa)	$(\overset{+}{H})$	
....	0.0009N	0.0920N	2×10^{-7}N	p-Nitrophenol
....	0.0023N	0.0920N	5×10^{-7}N	
....	0.0046N	0.0920N	1×10^{-6}N	
....	0.0092N	0.0920N	2×10^{-6}N	
....	0.0230N	0.0920N	5×10^{-6}N	
....	0.0460N	0.0920N	1×10^{-5}N	
....	0.0920N	0.0920N	2×10^{-5}N	

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[1] L. J. Henderson: *Biochemische Zeitschrift*; xxiv, p. 40, 1910.

amount of the acid which is free in solution, and the relative amount which is in combination with base. The ionisation constants of the important urinary acids have now been determined.[1] These quantities are as follows.

Hippuric acid	0.00022
Acetoacetic acid	0.00015
Lactic acid	0.00013
β-Oxybutyric acid	0.00002
Uric acid	0.0000015
Carbonic acid	0.0000003
MH_2PO_4	0.0000002

From these data, with the help of the equations of the concentration law, the accompanying curves are constructed.

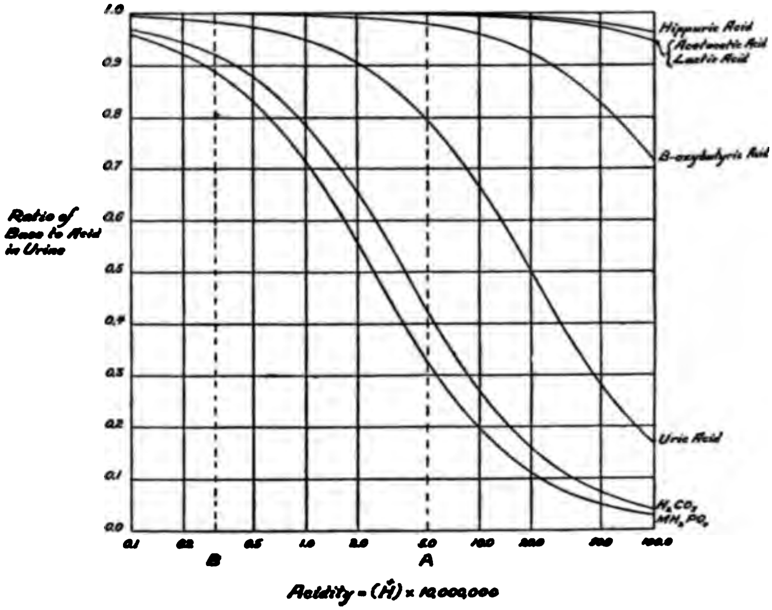

FIG. I. Reaction, expressed in terms of concentrations of ionized hydrogen, is plotted logarithmically on the axis of the abscissas. For convenience these concentrations are multiplied by 10,000,000. Ordinates represent the amount of base combined with one equivalent of acid. The lines *B* and *A* correspond to the reaction of the blood and to the mean reaction of the urine respectively. The several curves correspond to the urinary acids indicated.

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[1] For a discussion of this subject see Henderson and Spiro: *Biochemische Zeitschrift*, xv, p. 105, 1908.

These curves show the equilibria between acids and salts in such solutions as are found in urine. Evidently phosphoric acid, both on account of the magnitude of its avidity for base and on account of the relatively great amount present in urine is that one of the urinary acids which varies most in its ability chemically to bind base as the acidity of the urine changes within physiological limits. Thus chiefly through the presence of this substance the urine can carry out from the body varying amounts of acid in combination with a given amount of base. Accordingly it follows that phosphoric acid is primarily concerned, not only with the neutralization of acid in the body, but also with the preservation of the normal physiological mechanism of neutrality regulation by means of renal activity. For this task it possesses no less than ideal qualifications, as will be at once evident from a consideration of the actual intensity of the urinary acidity.

Quite recently measurements of the normal urinary acidity have been made in this laboratory in sufficient number to define the ordinary conditions of the equilibrium. These studies were carried out with indicators in urines diluted to such a degree that the normal color was nearly annulled and the influence of colored substances upon the indicator minimized. A series of solutions of known reaction was prepared in the customary manner by mixing varying quantities of acid and salt. The following table gives the constitution and the approximate reaction of these solutions.

No.	[illegible]	[illegible]	H	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	
	[illegible]	[illegible]	[illegible]	
1	[illegible]	[illegible]	[illegible]	
2	[illegible]	[illegible]	[illegible]	
3	[illegible]	[illegible]	[illegible]	
4	[illegible]	[illegible]	[illegible]	[illegible]
5	[illegible]	[illegible]	[illegible]	
6	[illegible]	[illegible]	[illegible]	
7	[illegible]	[illegible]	[illegible]	

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[1] L. J. [illegible]

Portions of these solutions were introduced into 250 cc. flasks and treated with an indicator, those having a hydrogen ion concentration greater than 2×10^{-7} N with paranitrophenol, the others with neutral red. The concentration of the indicator must be accurately fixed, and for these experiments the concentration of paranitrophenol was 0.08 per cent, the concentration of neutral red 0.0005 per cent. In carrying out the determinations 10 cc. of urine were placed in a 250 cc. flask, diluted with water, and treated with paranitrophenol. In case the color corresponded to that of one of the standard solutions the acidity was estimated either by noting that color with which the urinary sample corresponded, or in case the color lay between two standard colors, by rough interpolation. In case the acidity was less than that of the paranitrophenol solutions, a second portion of urine was treated with neutral red and the color compared with the more alkaline standard solutions. The results of such estimations are not the true values of the urinary acidity because dilution has slightly diminished the concentration of hydrogen ions. This change in equilibrium is not, however, an accidental one, and it amounts to a diminution in the concentration of hydrogen ions of approximately 25 per cent. The outcome of the investigation is that normal urine varies in concentration of ionized hydrogen from about 0.00000003N, the reaction of the blood, to about 0.000004N, though no doubt under exceptional conditions these limits may be surpassed. The mean value is about 0.0000005N. The results are in fairly good accord with measurements made by means of the concentration cell method. At any rate for the purpose of the present discussion there is no inconsistency whatever in existing data.

Such variations in acidity involve a change in the ratio of monosodium phosphate to disodium phosphate in the solution from 9 : 1 to 1 : 9 approximately, as indicated upon the curve. These variations represent, for such a range in the ratio of base to acid, the minimum possible variation in hydrogen ion content. Moreover the mean value of urinary acidity is such that in solution of that reaction the ratio of the two salts is not far from 1:1, and for such a ratio a change of whatever magnitude in the amount of acid or base involves the smallest possible change in acidity.[1]

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[1] For a mathematical discussion of these facts see L. J. Henderson· *American Journal of Physiology*, xxi, p. 173, 1908.

would be difficult indeed to account for this remarkable co-
lence, that phosphoric acid, one of the chief excretory pro-
:s of the urine, possesses the very highest possible efficiency in
physiological process for regulating the ratio of acids to bases
he body fluids by means of renal activity. Certainly there
ns to be nothing in evolutionary theory to explain it, and for
present it must be considered a happy chance, like the fact
the maximum density of water falls at 4°, and several other
cally similar circumstances.

phosphoric acid be the chief substance which is varying in the
unt of base associated with it as the reaction of the urine varies,
none the less true that other substances are varying too. How-
, only two, so far as we now know, will at the ordinary physio-
al ranges show considerable change in combined base. These
tances are carbonic acid and uric acid, both of which occur in
small amount as to be of minor importance. At the mean
e of the normal urinary acidity, while phosphates are present
it a third as monosodium phosphate and two thirds as disodium
phate there is nearly an equal amount of free carbonic acid
bicarbonate and about four times as much sodium urate as
uric acid.

s the acidity of urine increases into the unknown values char-
ristic of acid intoxication another variable factor appears,
ely β-oxybutyric acid. This substance, seldom appreciably,
r more than 10 per cent or 15 per cent free in urines of normal
tion, rapidly gives up its base as the acidity increases from this
t. And if it be possible for the kidney to produce a urine in
h the hydrogen ion concentration instead of being about one
dred times that of blood, which is not an uncommon value, is
it one thousand times that of blood, then β-oxybutyric acid
ld be almost wholly free. Evidently such a circumstance
ld be of extreme importance in diabetes, and would involve
mall change in current ideas of the proper treatment of dia-
c acidosis. This subject has been discussed elsewhere by
o and myself.[1]

the light of these considerations it is evident that there are
ranges of acidity in which small variations of reaction are,

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[1] enderson and Spiro: *loc. cit.*

or may be, accompanied by large changes in the ratio of base to acid in urine. The one range is centered upon the hydrogen ion concentration 2.5 × 10^{-7}N, and its importance is due to the fact that at this point the ratio of monosodium phosphate to disodium phosphate, here equal to one, is susceptible to little change as acid and alkali vary in amount. The other range is centered upon the hydrogen ion concentration 2.5 × 10^{-5}N. At this reaction β-oxybutyric acid and its salts are present in equivalent amounts, and accordingly a large change in the amount of acid or salt will have relatively little effect upon this ratio and therefore upon the acidity.

It is not to be expected, however, that all possible changes in reaction of urine will depend solely upon these two acids. A variety of other acid substances must each take part in a limited degree in such a change, thereby producing in the sum an appreciable effect. The actual conditions can best be illustrated by experiment. Following is the report of an experiment chosen from among several similar concordant experiments which well illustrate the relationships.

A sample of normal urine was divided into two portions, to one portion a small quantity of synthetic β-oxybutyric acid, enough to make the concentration of the acid about $\frac{1}{10}$ molal, was added, the two portions were diluted to $\frac{1}{10}$ of the original volume, and to each tenth-normal hydrochloric acid was poured in until the reaction, indicated by methyl orange, corresponded to a concentration of hydrogen ions, 1 × 10^{-4} N. Numerous samples of these two fractions were then titrated to several fixed stages of acidity and alkalinity. In the titrations 125 cc. portions of diluted urine, 0.115 N sodium hydrate, and either methyl orange, or p-nitrophenol, or neutral red, as indicator, were employed. Reaction was estimated by comparison of colors with those of indicator solutions containing phosphate and acetate mixtures of known acidity. The results of titration are shown in the accompanying table. The concentration of phosphoric acid in the original undiluted urine was approximately 0.068 molal. Differences in concentration preclude the simultaneous discussion of several experiments. The results of four similar experiments are, however, wholly concordant with those reported in the accompanying table.

DICATOR		NEUTRAL RED				PARA-NITROPHENOL					METHYL ORANGE
$(\overset{+}{H})$		2×10^{-8}	4×10^{-8}	1×10^{-7}	2×10^{-7}	4×10^{-7}	1×10^{-6}	2×10^{-6}	2×10^{-6}	1×10^{-5}	4×10^{-5}
centimeters diluted urine	I. Normal Urine	5.72	5.50	5.00	4.55	3.70	3.21	2.80	2.40	1.89	0.40
		6.10	5.66	5.10	4.70	3.53	3.12	2.70		1.80	0.50
		6.02	5.79	5.15	4.70	3.61	3.23	2.84	2.61	1.61	0.40
		6.30	5.82	5.36	4.75	3.80	3.17	2.96	2.61	1.60	0.32
	II. Urine + β-oxy-butyric Acid	6.93	6.70	6.02	5.50	5.00	4.37	4.10	3.61	2.83	0.43
		7.41	7.00	6.22	5.60	4.72	4.40	3.90	3.70	2.61	0.40
		7.50	7.10	6.40	5.70	4.60			3.60	2.68	
		7.40	6.98	6.20	5.50	4.80	4.12	3.70	3.50	2.57	
	Averages I	6.03	5.69	5.15	4.67	3.66	3.18	2.82	2.54	1.72	0.40
	Averages II	7.31	6.95	6.21	5.57	4.78	4.29	3.90	3.60	2.67	0.41
	Difference	1.28	1.26	1.06	0.90	1.12	1.11	1.08	1.06	0.95	0.01

Two facts are at once visible in the data.

(I) When alkali is poured into acidified normal urine the greater rt of it is neutralized only after the reaction has become nearly utral $[(\overset{+}{H}) = 2 \times 10^{-6}N]$ in that range of reaction

$$[2 \times 10^{-6}N > (\overset{+}{H}) > 2 \times 10^{-8}N]$$

hich is the domain of mixtures of monosodium phosphate and sodium phosphate, and is characteristic of normal human urine.

(II) In the titration the neutralization of β-oxybutyric acid is on approximately complete

$$[(\overset{+}{H}) = 1 \times 10^{-5}N]$$

d thereafter the neutralization of urine to which it has been dded progresses, so far as measurements with indicators can ow, exactly like the neutralization of normal urine.

Further analysis of the data clearly shows that phosphates alone d unaided are, within the limits of accuracy of the measureents, wholly responsible for all neutralization in the neighborhood of the neutral point. The accompanying diagram, fig. 2, makes clear the basis of this conclusion.

The curve represents diminution in amount of alkali (corresponding to alkali retention or acid excretion) as the concentration

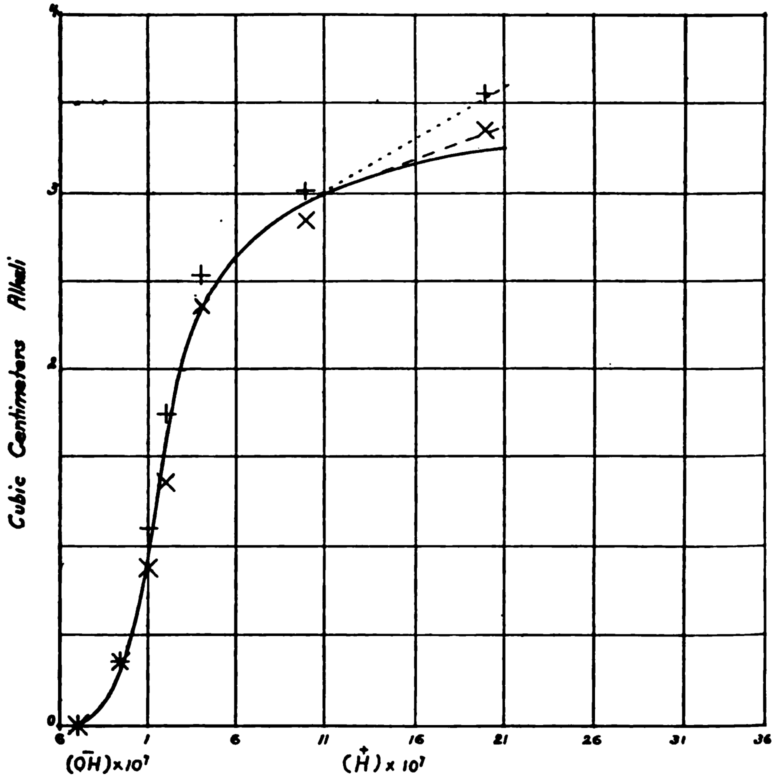

FIG. II. Gradual Titration of Urine. *Abscissas* stand for alkalinity and acidity plotted arithmetically to show proportional changes.

Ordinates stand for cubic centimeters of alkali.

×, titration of normal urine.

+, titration of normal urine plus β-oxybutyric acid.

The curve represents theoretical values for the titration of the phosphates of the urinary sample.

of ionic hydrogen increases from 2×10^{-8}N [$(\bar{OH}) = 5 \times 10^{-7}$N] in a phosphate solution of the same concentration, 0.0034 molal, as the phosphates in the diluted urinary sample. The correspond-g quantities as measured by titration of the normal urine and the ine plus β-oxybutyric acid are indicated by crosses. The line dashes corresponds to the titration of normal urine, the dotted ne to the titration of urine plus β-oxybutyric acid. Hydrogen d hydroxyl ion concentration are plotted as abscissas, "acid cretions" as ordinates. The incidence of the numbers derived om the data upon the theoretical curve leaves no room for doubt at within the normal ranges of urinary reaction phosphates one are measurably responsible for the variation in ratio of acid d base. A more or less precise formulation of this fact has long ssed current, but it has not before been experimentally demonrated.

Repeated experiments like that above reported with numerous mples of normal urine have invariably shown that only near the action of normal urine does that solution possess marked capacity resist change of reaction. Within that range it behaves, so r as titration can show, exactly like its phosphate content. That to say, the titration of the phosphates alone, of urine, and of rine containing β-oxybutyric acid, within the limits of acidity of rmal urine, are based upon the same chemical changes;—conrsion of alkaline phosphates into acid phosphates according to e reaction, $NaH_2PO_4 + NaOH = Na_2HPO_4 + H_2O$.

In order to consider the phenomena at higher ranges of acidity it desirable to plot the data in another manner, employing for scissas the logarithms of the concentrations of ionized hydrogen, in Fig. III.

A study of this figure, which presents in convenient form, the m of the effects of variations in relative amounts of acid and se in urine upon its reaction, clearly shows the conditions which eory predicts. Like the phosphates, the β-oxybutyrates are fective in neutralization only in the immediate neighborhood of hydrogen ion concentration corresponding to the ionization conant of β-oxybutyric acid (2×10^{-5}). But at this point, unlike e point at which phosphates neutralize, other substances are lso concerned in the process, and the change in ratio of acid base is the sum of many similar processes, among which change the β-oxybutyrate equilibrium is merely the most important.[1]

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[1] The apparent change shown by titration is somewhat too great at this int because of dilution of the urine. The error however, is small, and, in e present discussion, negligible.

It needs no intricate analysis of the facts here figured to make clear the one important practical outcome of the experiment. Possible variations in the amount of base combined with each and all of the constituents of normal urine, except only phosphoric acid, are, in the healthy, normal organism, relatively unimportant.

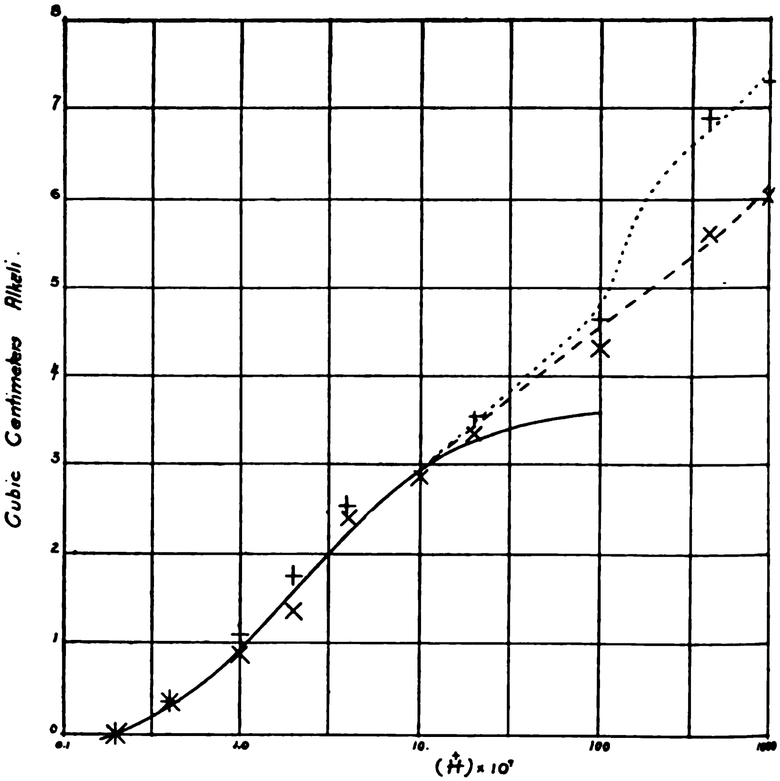

Fig. III. Gradual Titration of Urine. *Abscissas* stand for acidity $[(\overset{+}{H}) \times 10^7]$ plotted logarithmically. Otherwise the figure corresponds to Fig. II.

Phosphoric acid, with carbonic acid, is the principal factor in the adjustment of the reaction of the body fluids; with ammonia it is the principal factor in the permanent maintainance of that reaction through the regulatory activity of the kidney.

SUMMARY.

Like temperature and osmotic pressure, the normal neutrality or alkalinity $[(\overline{\mathrm{O}}\mathrm{H})_{38^{\circ}} = 7 \times 10^{-7}\mathrm{N}]$ is adjusted by a mechanism within the body, but permanently maintained by exchanges with the environment.

The acid end products of metabolism, without appreciably changing the actual alkaline reaction constantly take up alkali from blood and protoplasm. In this manner there is a tendency to disturb the normal protective equilibrium between bases and acids. This tendency is held in check by the kidney, which in the process of urine formation reverses the reaction of neutralization of acid and restores to the blood that alkali which has served as a carrier of acid.

The process may be measured both quantitatively, and in respect to its efficiency. The quantity of acid excreted is measured by the amount of alkali which must be added to urine in order to obtain the reaction of blood plus the amount of urinary ammonia.

The method of carrying out this measurement, as devised by Adler and myself, is described.

The efficiency of acid excretion depends upon the intensity of acidity of the urine; upon whether a large or a small quantity of acid is there present in addition to that quantity which, in the blood was associated with the urinary bases. The efficiency may be estimated by measuring the concentration of ionized hydrogen, but is not proportional to this quantity. Equal increments of increase in acidity are associated with variable increments of increase in acid excretion because of the particular avidities of the several urinary acids, especially acid sodium phosphate and β-oxybutyric acid. Phosphoric acid is as well suited to facilitate the excretion of acid as to regulate the neutrality of the body. The strength of acid sodium phosphate as an acid is precisely that which permits the widest range in the ratio of base to acid within the ordinary limits of urinary acidity.

Experiments are reported in which gradual titrations of normal urine and of urine containing β-oxybutyric acid have been carried out. These experiments show that normal variations in the reaction of urine are almost wholly due to variation in the ratio of alkaline to acid phosphates excreted by the kidney.

Accordingly the preservation of the normal alkalinity of the body is due, in important measure, to but two independent factors, excretion of ammonia and excretion of phosphates. The elaboration of ammonia and the presence of phosphoric acid as an end product of metabolism make possible the excretion of acid; regulation of ammonia production and of the ratio of the acid to the alkaline phosphates of urine are the means of regulating the acid excretion.

NALYSIS OF THE PRODUCTS OF HYDROLYSIS OF WHEAT GLIADIN.[1]

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though analyses of the products of hydrolysis of gliadin from t have already been made by Abderhalden and Samuely[2] and by Osborne and Clapp[3] it has seemed to us desirable to make w analysis of this protein because experience with the analy- methods has indicated that a much higher summation and re complete knowledge of the proportion of the several amino- s can now be obtained.

e unique constitution of gliadin has already led to its use in y nutrition investigations and the ease with which it can be ared in a relatively pure condition renders it one of the most lable proteins for further experiments. As complete a knowl- as possible of its products of hydrolysis is consequently essen- for future studies of physiological problems.

nce the fact has become apparent that in the earlier analyses roteins were not boiled with acid long enough to effect com- hydrolysis we have followed the progress of the hydrolysis iadin by determining, from time to time, the proportion of o nitrogen by the method of VanSlyke.[4] We thus found that boiling with hydrochloric acid, sp. gr., 1.1, for forty-eight s no further increase in the amount of amino nitrogen occurred,

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he expenses of this investigation were shared by the Connecticut ultural Experiment Station and the Carnegie Institution of Washing- D. C.

bderhalden and Samuely: *Zeitschr. f. physiol. Chem.*, xliv, p. 276, 1905.

sborne and Clapp: *Amer. Jour. of Physiol.*, xvii, p. 231, 1906

anSlyke: *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3170, 1910; this *al*, ix, p. 184, 1911.

TE: In carrying out this operation we have employed a somewhat ent form of apparatus than that used by VanSlyke but the essential res of the process were practically the same.

and also that practically all of the nitrogen, which escaped isolation in definite form, is amino nitrogen. Thus if the nitrogen which is not estimated as amino nitrogen by VanSlyke's method is calculated from the results of our new analysis we find the partition of nitrogen to be as follows:

	PER CENT OF NITROGEN	PER CENT OF GLIADIN
Ammonia nitrogen	24.57	4.30
Amino nitrogen	59.20	10.36
Proline nitrogen	9.12	1.60
Three-fourths arginine nitrogen	4.36	0.76
Two-thirds histidine nitrogen	0.60	0.11
	97.85	17.13

A part of the nitrogen thus unaccounted for belongs to tryptophane which the Hopkins-Cole reaction shows to be present among the products of hydrolysis of gliadin but which cannot be estimated quantitatively.

The results of our new analysis of gliadin, together with the earlier analysis of Osborne and Clapp are as follows:

	NEW ANALYSIS	OSBORNE AND CLAPP	COMBINATION OF THE HIGHEST RESULTS
Glycocoll	0.00	0.00	0.00
Alanine	1.95	2.00	2.00
Valine	3.34	0.21	3.34
Leucine	6.62	5.61	6.62
Proline	13.22	7.06	13.22
Phenylalanine	1.80	2.35	2.35
Glutaminic acid	43.66	37.33	43.66
Aspartic acid	0.14	0.58	0.58
Serine		0.13	0.13
Tyrosine		1.20	1.20
Cystine		0.45	0.45
Histidine		0.61	0.61
Arginine		3.16	3.16
Ammonia	5.22	5.11	5.22
Tryptophane, about		1.00[1]	1.00
		66.80	83.54

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[1] Abderhalden: *Zeitschr. f. physiol. Chem.*, xliv, p. 276, 1905.

It is thus seen that the yield of valine, leucine, proline and glutaminic acid has been very materially increased by the prolonged hydrolysis while the amounts of alanine, phenylalanine and aspartic acid are about the same as found in the earlier analysis. If the higher results, which, in our opinion, are the most nearly correct, are added together the total of the amino-acids found is equal to 83.54 per cent of the gliadin.

Assuming these amino-acids to be combined in polypeptide union, and ammonia as substituting one hydroxyl of the dibasic acids, we have a total of 67.90 per cent of the gliadin accounted for. A part of the deficit of 32.10 per cent is certainly due to unavoidable losses incurred in estimating those amino acids which are obtained from their esters. If such losses corresponded to those found by Osborne and Jones[1] in thus analyzing a mixture of pure amino-acids the total *probable* proportion of the radicals of the amino-acids stated in the analysis becomes 82.14 per cent of the gliadin. In making this estimate it is assumed that only one-half of the alanine, aspartic acid, serine, cystine, and tryptophane, 70 per cent of the valine, proline, phenylalanine, and glutaminic acid obtained from the esters, 80 per cent of the leucine and all of the histidine, arginine, and ammonia, and also all of the glutaminic acid separated directly, were recovered.

In view of past experience such an estimate seems conservative and the assumption appears justified that at least 82 per cent of the gliadin consists of radicals of the substances enumerated in the analysis.

The following table compares the results of this analysis of gliadin with similar analyses of zein and casein made with a view to obtain the highest possible yields of each of the amino-acids.

Comparison of the results of analysis of casein, gliadin and zein.

	TOTAL AMINO-ACIDS ACTUALLY RECOVERED	EQUIVALENT RADICALS	PER CENT OF NITROGEN RECOVERED	ESTIMATED PROBABLE QUANTITY OF RADICALS	PER CENT OF THE TOTAL N IN ESTIMATED RADICALS
Casein....	67.85	57.45	63.60	70.37	75.6
Gliadin...	83.54	67.90	75.70	82.14	87.2
Zein.......	88.87	71.37	80.50	88.72	95.9

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[1] Osborne and Jones: *Amer. Journ. of Physiol.*, xxvi, p. 305, 1910.

These figures show that from each of the three proteins the recovered nitrogen forms a larger proportion of the total nitrogen than the recovered radicals form of the total protein. This is chiefly due to the fact that the nitrogen of the basic amino-acids and ammonia are practically completely recovered, the mean nitrogen content of which is relatively large, thus for gliadin it is equal to 66.2, for zein to 67.4, and for casein to 35.5 per cent of the radicals.

If the recovered basic radicals and ammonia, (calculated as NH_2), are deducted from the total recovered radicals we find that the per cent of the protein recovered as mono-amino-acid radicals is as follows:

	PER CENT OF PROTEIN
Casein	45.15
Gliadin	59.53
Zein	65.78

The recovered mono-amino-acids form a slightly larger proportion of the non-basic part of the protein as shown by the following figures:

Recovered mono-animo radicals as per cent of the non-basic part of the protein.

Casein	48.5
Gliadin	64.9
Zein	69.6

The following figures give the mean per cent of nitrogen in the recovered mono-amino-acids and in the unrecovered non-basic part of the proteins.

Percent of nitrogen in non-basic parts.

	RECOVERED	UNRECOVERED
Casein	12.3	13.3
Gliadin	13.0	13.1
Zein	13.9	10.9

These figures show that the unrecovered part of casein and gliadin contains about the same proportion of nitrogen as that in the recovered part and indicate that the deficit is chiefly caused by an incomplete recovery of the amino-acids already known to result from the decomposition of these proteins. The much lower nitrogen content of the undetermined part of zein suggests the presence of some undiscovered radical containing but little, if any, nitrogen.

In these analyses the proportion of the recovered radicals, as well as of the nitrogen, decreases with increasing complexity of the protein. This is evidently not to be explained by a correspondingly large proportion of radicals which are determined without the use of the ester method, namely of glutaminic acid, tyrosine, arginine, histidine, lysine, and ammonia, for the sum of these from gliadin is 42.78 per cent, whereas, from casein it is only 29.81, and from zein 26.68 per cent, proportions which stand in no relation to the total amounts of decomposition products recovered from these three proteins.

If the amount of those radicals, which are estimated directly, is subtracted from the total radicals recovered we find that from casein 27.64, from gliadin 25.07, and from zein 44.69 per cent of the protein were recovered as radicals by the ester method. We thus find that for casein 39.3, for gliadin 43.8, and for zein 60.9 per cent of the total non-basic part of the protein were recovered by the ester method. The proportion thus recovered from zein is much greater than from casein and gliadin, a result in part due to the absence of tryptophane and also to the relatively large amount of leucine yielded by zein. It is possible that casein and gliadin actually yield relatively considerable quantities of serine, oxyproline or aspartic acid only a small part of which were recovered in the analysis. Tryptophane unquestionably contributes to a considerable extent to this deficit but whether or not enough is present to account for most of the very considerable deficit it is impossible at present to determine. These considerations show the importance of discovering some method whereby an even approximate determination of tryptophane can be made.

EXPERIMENTAL PART.

A quantity of very carefully purified gliadin, equivalent to 459.8 gm. of ash- and moisture-free protein, was divided into five equal parts and hydrolyzed by boiling each separately with 200 cc. of hydrochloric acid, sp. gr. 1.1, for forty-eight hours. The hydrolysis solutions were then diluted, and separately filtered, in order to remove the small amount of humin which they contained. The total humin from all of the five portions weighed 1.4 gm. and contained 0.095 gm. of nitrogen. After filtering out the humin each solution was made up to one liter with water and 10 cc. taken from each, diluted to 100 cc., and total nitrogen determined in an aliquot of 10 cc. The amino nitrogen was also determined by VanSlyke's method in portions of 25 cc. The total nitrogen thus estimated as present in each of the five solutions was the same, namely 16.09 gm.; the amino nitrogen in each was practically the same, the average being 9.53 gm. The amino nitrogen was thus equal to 59.2 per cent of the total nitrogen. Each solution was then concentrated and brought to a volume of about 200 cc. by adding more hydrochloric acid. After boiling for twenty-four hours longer no further separation of humin occurred and the percentage of amino nitrogen was not changed. The amount of substance removed for these determinations was equal to 2 per cent of the gliadin originally taken, hence that which remained corresponded to 450.6 gm. on the basis of which the percentage yields of that part of the glutaminic acid which was determined directly, was calculated.

The solutions were then concentrated, saturated with hydrochloric acid, and glutaminic acid hydrochloride allowed to crystallize after standing in an ice chest. The filtrates were concentrated and a second crop obtained. These two crops of glutaminic acid hydrochloride from each of the five solutions were united, decolorized, and separately recrystallized. They weighed respectively 35.96, 29.73, 34.49, 34.42, and 36.68 gm. The united mother liquors from the recrystallization of these several crops were concentrated and 31.87 gm. more were secured, making 203.15 gm. of glutaminic acid hydrochloride separated by direct crystallization, equal to 162.66 gm. of free glutaminic acid or 36.1 per cent of the gliadin. There were subsequently obtained from the esters 33.82 gm. of free glutaminic acid equal to 7.56 per cent of the gliadin, making 43.66 per cent in all.

This result is much higher than the 37.33 per cent formerly obtained by Osborne and Harris[1] and later confirmed by the closely agreeing results of Abderhalden and Samuely.[1] It can however be accepted as a minimal percentage, for especial care was taken to weigh only pure products, and another determination, made after a correspondingly long hydrolysis, gave almost as high a result, namely 43.19 per cent. In this case also the glutaminic acid hydrochloride was subjected to a very critical examination and its purity established beyond question.[2] The amount of glutaminic acid yielded by gliadin is probably even greater, for, in our present analysis 7.56 per cent was obtained from the esters and if the amount obtained from the esters was 70 per cent of that actually present[2] this would correspond to 10.7 per cent, making the probable total at least 47 per cent. Even this amount is probably too low for the total glutaminic acid hydrochloride which was obtained by direct crystallization, before decolorizing with bone black weighed 275.01 gm. Analysis showed that this contained 5.1 gm. of moisture and 44.68 gm. of ammonium chloride. It contained chlorine corresponding to a mixture of this amount of ammonium chloride and practically pure glutaminic acid hydrochloride, for, deducting the ammonium chloride the remainder contained chlorine equal to 19.53 per cent, as against 19.34 per cent, calculated for pure glutaminic acid hydrochloride. Deducting the weight of the ammonium chloride we have 225.23 gm., equal to 180.48 gm. of free glutaminic acid, which is 17.82 gm. more than the 162.66 gm. of pure acid obtained after decolorizing with bone black. The loss thus indicated is equal to 3.96 per cent of the gliadin, which, if added to the estimated 47 per cent makes a total possible yield of about 51 per cent.

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[1] Osborne and Harris: *Amer. Journ. of Physiol.*, xiii, p. 35, 1905.

Further evidence that gliadin actually yields a larger proportion of glutaminic acid than the total which we obtained in a perfectly pure condition is afforded by the fact that one of the five solutions from which the glutaminic acid hydrochloride was directly separated yielded a quantity of pure hydrochloride equal to 45.34 per cent.

The filtrates from the glutaminic acid were united, the solution concentrated, under diminished pressure, and, after removing the water by repeated evaporation with alcohol, the amino-acids were esterified according to the method of Phelps and Phelps. The esters were liberated by a calculated quantity of sodium ethylate, the sodium chloride was separated from the alcoholic solution of the esters by decantation and filtration. This solution was then evaporated under diminished pressure, made up to a volume of two liters and found to contain 38.192 gm. of nitrogen.[1] The sodium chloride contained 0.72 gm. of nitrogen. The alcoholic solution of the esters was concentrated under diminished pressure until nearly all the alcohol was removed. The alcoholic distillate from the free esters was acidified with sulphuric acid and evaporated under diminished pressure to a small volume. The sulphuric acid was then removed with baryta and after evaporating to dryness the residue was extracted with alcohol and 1.74 gm. of amino-acids insoluble in alcohol were obtained. The alcoholic extract of this residue contained 2.5659 gm. of nitrogen, of which 0.7897 gm. was found to be amino nitrogen, leaving 1.7762 gm. of non-amino nitrogen, equivalent to 14.65 gm. of proline, equal to 3.28 per cent of the gliadin. The crude esters, which weighed 450 gm., were distilled under diminished pressure, as follows:

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[1] Abderhalden and Samuely: *Zeitschr. f. physiol. Chem.*, xlvi, p. 193, 1905.

[2] Osborne and Jones: *Amer. Journ. of Physiol.*, xxvi, p. 305, 1910.

Distillation I

FRACTION	TEMPERATURE OF THE BATH UP TO	PRESSURE	WEIGHT
		mm.	*gm.*
I	97°	3	55.3
Condensed in liquid air tube 1.			31.0
II	100°	1	60.0
Condensed in liquid air tube 2.			23.0
III	115°	1	15.0
Condensed in liquid air tube 3.			17.0
IV	175°	1.7	25.0
Condensed in liquid air tube...			28.0
Undistilled residue			187.0
			441.3

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[1] In thus estimating nitrogen and the hydrochloric acid in this solution an aliquot part was removed equivalent to 3 gm. of the original gliadin. The percentages of the amino-acids obtained from these esters were therefore calculated on the basis of 447 gm. of gliadin.

The solutionss condensed in liquid air tubes 1, 2, and 3 were united and saponified with baryta. After removing the baryta 1.12 gm. of substance was obtained which was added to fraction I.

Fraction I was saponified in the usual way and the amino-acids extracted thoroughly by boiling with absolute alcohol. The amino-acids insoluble in alcohol weighed 18.2 gm. The alcoholic extract was added to that similarly obtained from fractions II and III.

Fraction II yielded 26.43 gm. of amino-acids insoluble in alcohol.

Fraction III was saponified with baryta and yielded 3.78 gm. of amino-acids insoluble in alcohol from which 1.33 gm. of copper aspartate, equal to 0.64 gm. of aspartic acid were obtained. The rest of the amino-acids was added to the substance from fraction II which was insoluble in alcohol.

The united alcoholic extracts from fractions I, II, and III contained 5.2920 gm. of nitrogen of which 0.5410 gm. was found to be amino nitrogen. The non-amino nitrogen was therefore 4.7510 gm. equal to 39.04 gm. of proline or 8.73 per cent. This together with the 3.28 per cent obtained from the alcohol distilled from the free esters and 1.21 per cent separated from the distillation residue makes 13.22 per cent of proline.

Fraction IV was mixed with an equal volume of water and the phenylalanine ester extracted as usual with ether. There were obtained 6.12 gm. of phenylalanine hydrochloride equal to 5.01 gm. of phenylalanine or 1.12 per cent. From the aqueous solution by the usual treatment 2.85 gm. of glutaminic acid hydrochloride equal to 2.28 gm. of the free acid or 0.5 per cent of the gliadin was separated. From the mother liquor from the glutaminic acid hydrochloride, no aspartic acid or serine was obtained.

DISTILLATION RESIDUE.

The distillation residue contained 19.5 gm., of nitrogen or about 25 per cent of the total nitrogen of the gliadin. In order to determine what amino-acids this contained it was dissolved in concentrated hydrochloric acid and boiled for fifteen hours in order to hydrolyze such esters, or peptides which it contained, as well as anhydrides, which had been formed from the esters during the distillation. Glutaminic acid was isolated as the hydrochloride and 22.85 gm. obtained, equal to 18.31 gm. of the free acid, or 4.09 per cent of the gliadin.

The filtrate from the glutaminic acid hydrochloride was diluted to 6 liters with water and 1 liter of a solution containing 200 gm. of phosphotungstic acid, dissolved in 5 per cent hydrochloric acid, was added. The filtrate from the precipitate thus produced gave no more precipitate on the addition of phosphotungstic acid. The precipitate was decomposed with baryta and, after removing phosphotungstic and sulphuric acids the arginine and histidine were separated according to the method of Kossel and Patten. Only 1.1 gm. of histidine, equal to 0.25 per cent of the gliadin, and 3.77 gm. of arginine, equal to 0.85 per cent of the gliadin, were obtained. It thus appears that during the process of distillation a very large part of these bases was destroyed. The filtrate from the phosphotungstic acid precipitate was freed from the excess of phosphotungstic and sulphuric acids, concentrated to a syrup, and saturated with hydrochloric acid. After standing on ice 6.07 gm. of glutaminic acid hydrochloride, equal to 4.86 gm. of glutaminic acid, or 1.09 per cent of the gliadin, separated. The amino-acids in the mother liquor from the glutaminic acid were then esterified by the method of Phelps and Phelps and the solution of the esters made up to 500 cc. and found to contain 10.388 gm. of nitrogen equal to 12.9 per cent of the total nitrogen of the gliadin. After liberating the esters with sodium ethylate the sodium chloride was separated by centrifugation, and, when washed with alcohol as thoroughly as possible, contained 0.453 gm. of nitrogen. The alcohol was distilled from the esters under diminished pressure at 40° and the residue treated with ether which caused the separation of a semi-solid mass which contained 2.15 gm. of nitrogen. The ether was distilled from the solution of the esters and the residual esters distilled as follows:

Distillation II.

FRACTION	TEMPERATURE OF BATH UP TO	PRESSURE	WEIGHT
		mm.	*gm.*
I	115°	1.8	39
II	185°	1.8	17
Undistilled residue			17

Fraction I yielded 4.5 gm. of amino-acids insoluble in alcohol, and an alcoholic extract which contained 1.1564 gm. of nitrogen, of which 0.4930 gm. was amino nitrogen, leaving 0.6634 gm. of non-amino nitrogen equal to 5.45 gm. of proline or 1.21 per cent of the gliadin.

Fraction II gave 3.7 gm. of phenylalanine hydrochloride, equal to 3.03 gm. of phenylalanine, or 0.67 per cent of the gliadin and 10.45 gm. of glutaminic acid hydrochloride, equal to 8.37 gm. of glutaminic acid, or 1.87 per cent of the gliadin. This examination of the distillation residue showed that one-third of the nitrogen belonged to amino-acids which had not been completely isolated in the earlier steps of the analysis, probably largely because, in the process of distillation, a considerable part of these were condensed to dioxopiperazines.

From the amino-acids insoluble in alcohol from Distillation I, fractions I, II, and III, and from Distillation II, fraction I, by using the lead method of Levene and VanSlyke, 29.6 gm. of leucine, equal to 6.62 per cent of the gliadin, 14.96 gm. of valine, equal to 3.34 per cent of the gliadin and 8.74 gm. of alanine, equal to 1.95 per cent of the gliadin, were obtained.

The distribution of the nitrogen among the various products obtained in the above described process is shown in the following table:

	GRAMS	PER CENT
Total nitrogen in the gliadin	80.36	
Nitrogen in glutaminic acid, separated directly as pure hydrochloride	15.50	19.29
Nitrogen in humin	0.10	0.12
Nitrogen as ammonia	19.35	24.08
Nitrogen in the ester hydrochlorides	40.32	50.17
Nitrogen unaccounted for	5.09	6.34
	80.36	100.00

The nitrogen unaccounted for is to be ascribed in part to loss in decolorizing the glutaminic acid hydrochloride, for, as already shown, a loss of glutaminic acid equal to 2.1 per cent of nitrogen appears to have occurred.

In the above table one-half of the total nitrogen of the gliadin appears as present in the hydrochlorides of the esters. Of the 40.32 gm., 17.96 gm. was recovered in the alanine, valine, leucine. proline, phenylalanine, glutaminic acid, and aspartic acid, which were subsequently separated from the esters. Of the remaining 22.36 gm., 6.80 gm. have been shown by direct determinations to belong to tyrosine, serine, cystine, histidine, arginine, and tryptophane, thus leaving 15.56 gm. or 38.59 per cent of the ester nitrogen unaccounted for. We have thus by a combination of the ester method and direct methods accounted for 61.4 per cent of the nitrogen present in the solution of the ester hydrochlorides. Considering the well known limitations of these methods, especially those for estimating tryptophane, serine, and cystine, the unavoidable losses are unquestionably large, but whether these losses are sufficient to account for all of this deficit must be determined by future investigations.

As to the location of these losses we have obtained the following data. The esters were liberated with sodium ethylate from the solution of the hydrochlorides and 32.5 gm. of the 40.32 gm. originally present were found in the alcoholic solution of the free esters. The sodium chloride and other substances insoluble in alcohol were extracted with alcoholic hydrochloric acid and the extracted substances again esterified. The alcoholic solution of the esters thus obtained contained 5.71 gm. of nitrogen, hence 38.21 gm. were recovered in the two alcoholic solutions of the free esters, or 94.7 per cent of the original 40.32 gm. contained in the solution of the ester hydrochlorides. The loss in liberating the esters by sodium ethylate was therefore relatively small.

These esters were distilled and the amino-acids isolated as already described. In the amino-acids recovered in definite form there were 13.905 gm., in the alcoholic solution of the proline 1.419 gm., and in the undistilled residue 19.5 gm. of nitrogen, making 34.824 gm. in all. The 3.386 gm. of nitrogen thus unaccounted for was chiefly present in substances in the upper fraction containing the phenylalanine, aspartic acid and glutaminic acid esters. This unidentified substance doubtless contained some serine together with volatile secondary decomposition products of the various esters which composed the original mixture.[1] The distilled esters, therefore, contained as unidentified nitrogen the sum of the unidentified nitrogen of the higher boiling fractions and the amino nitrogen of the alcoholic solutions of the proline, or 4.805 gm., equal to 12.5 per cent of the total nitrogen contained in the original alcoholic solution of the ester hydrochlorides.

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[1] Cf. Osborne and Jones: *Amer. Journ. of Physiol.*, xxvi, p. 305, 1910.

About one-half, or 19.5 gm., of the nitrogen of the esters soluble in alcohol remained in the distillation residue. This was treated as already described, p. 433, and yielded 4.09 gm. of nitrogen in amino-acids isolated in definite form. If we assume that all the nitrogen of the arginine, histidine, tyrosine, and tryptophane, equal to 6.48 gm., as determined by direct isolation, remained in this undistilled residue, the total amount, which might thus be possibly accounted for is 10.57 gm., leaving 8.93 gm. or 23.4 per cent of the total nitrogen of the original esters

How much of the unidentified nitrogen of the distilled esters, which was 4.805 gm., resulted from decomposition of arginine, histidine, tyrosine, and tryptophane cannot be determined from any data we now possess further than that arginine and histidine esters apparently decomposed to a large extent during the distillation as shown by the fact that of the 5.44 gm. of nitrogen originally present in arginine and histidine only 1.59 gm. was recovered from this distillation residue, showing a loss of 3.85 gm. How much of this belonged to volatile decomposition products which distilled over with the esters cannot be estimated, but it is evident that this loss alone is insufficient to account for all of the unidentified nitrogen contained in the esters. However this may be, only 29.1 per cent of the nitrogen of this distillation residue was actually recovered as definite substances. These data show that the greatest loss occurred in distilling the esters and that this was equal to 16.4 per cent of the total nitrogen of the gliadin.

The losses in making this analysis can be thus summarized:

	GRAMS
In decolorizing the glutaminic acid hydrochloride solutions with bone coal	6.34
In separating the distilled esters	4.80
In the distillation residue	16.40

This statement indicates that 72.46 per cent of the total nit was recovered in definite form, whereas, in fact 75.7 per ce shown by the complete analysis to be accounted for. This ence is largely due to the decomposition of the bases, trypto and other amino-acids during the distillation.

SULPHUR LINKAGES IN PROTEINS.

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It is necessary to state at the outset that the above title is too broad for a single paper, so that only a small part of this field will be touched upon in this publication.

It has been known for a long time that sulphur is a constituent of protein, and that this element can be identified as lead sulphide by heating proteins with alkali in the presence of lead acetate. Not until about the year 1800, however, was chemical evidence of any importance produced, which indicated the nature of the sulphur groupings in these natural substances.

In 1797, Crawford[1] made his important and interesting observation, that hydrogen sulphide was evolved during the putrefacion of flesh. This was followed by Wallaston's discovery,[2] in 1810, of cystine in an urinary calculus and fourteen years later by Gmelin's[3] discovery of taurine in ox-gall. The first elementary analysis of taurine was made by Demarcay[4] in 1833. Pelouze and Dumas[5] later corroborated Demarcay's analyses and assigned o taurine the formula $C_2H_7O_3N$. Redtenbacher,[6] in 1846, demontrated that taurine contains sulphur, a fact which had been overooked by its discoverer and later investigators, and established its correct formula.

The first important observation, which led to any definite idea hat sulphur is linked in proteins in more than one way, was

[1] *Crells Annal.*, i, p. 335.

[2] *Phil. Trans.*, 1810, p. 223.

[3] Tiemann and Gmelin: *Die Verdauung.*

[4] *Ann. Chem. u. Pharm.*, xxvii, p. 286; *Chem. Zentrbl.*, 1838, p. 261.

[5] *Ibid.*, xxvii, p. 292.

[6] *Ibid.*, lvii, p. 170; lxv, p. 37.

made by Fleitmann[1] in Liebig's laboratory in 1846. He showed that only a part of the sulphur in certain proteins was removed by heating with alkali, and because of the similarity of their behavior to that of the two sulphur compounds already detected as products of animal metabolism—*cystine* and *taurine*—he designated the two forms as *oxidized* and *unoxidized* sulphur. Danielewski,[2] in 1869, confirmed Fleitmann's observations and in a later paper,[3] in 1883, called special attention again to them and their significance in computing the molecular weights of proteins.

Evidence that the terms *oxidized* and *unoxidized* sulphur did not truly represent the different types of sulphur groupings in these substances was produced by Krüger[4] in 1883. He determined carefully the relative proportions of the two forms of sulphur in ovalbumin and fibrin, and showed that mercaptans and thioethers behaved in a similar manner as these proteins when heated with alkali. On the other hand, oxidized sulphur compounds as sulfonic and sulfinic acids behaved quite differently and were decomposed, under the same conditions, giving alkali sulphites. Krüger therefore dropped the terms *oxidized* and *unoxidized* and designated the two forms as *loosely bound* and *firmly-bound* sulphur.

Goldmann and Baumann,[5] in 1888, showed that, by heating cystine with 10 per cent sodium hydroxide and lead acetate 68 per cent of the sulphur was removed as lead sulphide. Suter,[6] who later studied the action of alkali on proteins, observed the similarity of their behavior to that of cystine. He obtained, by increasing the time of heating with alkali, as high as 83 per cent of the sulphur in cystine.

Schülz[7] reviewed the work of these investigators and attributed their discordant results to oxidation of the sulphur during the digestion with alkali. In order to avoid this error he added zinc to the soda solution and then was able to account for only about 53 per cent of the sulphur in cystine after heating with 30 per cent alkali for twenty-five hours. Schülz also applied his method to several proteins.

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[1] *Ann. Chem. (Liebig)*, lxi, p. 121; lxvi, p. 380.

[2] *Zeitschr. f. Chem.*, xii, p. 41.

[3] *Zeitschr. f. physiol. Chem.*, vii, p. 443.

[4] *Pflüger's Archiv*, xliii, p. 243.

[5] *Zeitschr. f. physiol. Chem.*, xii, p. 257.

[6] *Ibid.*, xx, p. 564.

[7] *Ibid.*, xxv, p. 16.

Although the similarity between proteins and cystine, in their behavior towards alkali, was early recognized, nevertheless this knowledge had so far thrown little light on the nature of sulphur combinations in proteins. It was not until about 1895 apparently, when attention began to be paid by chemists to the hydrolytic sulphur cleavage products of proteins, that real significance became attached to these old observations.

Kulz,[1] in 1890, succeeded in isolating cystine from the pancreatic decomposition products of fibrin. This work was followed by the important announcement by Emmerling,[2] in 1894, of his discovery of this same substance among the decomposition products of horn. In 1896, Drechsel[3] observed the formation of a volatile sulphide by hydrolysis of a keratin with hydrochloric acid, and believed it to be identical with ethylsulphide, which Abel[4] had detected the year before in urine. He also observed that the substance from which this volatile sulphide was formed was a base, which was precipitated by phosphotungstic acid. This basic substance however was not identified. A year later Müller and Seemann[5] also described a substance, containing sulphur, of unknown constitution, which was obtained as a cleavage product of egg-white. Similar observations were made by Blum and Vaubel[6] and also by Hedin.[7]

In 1899, appeared Mörner's important paper entitled "Cystin, ein Spaltungsprodukt der Hornsubstanz."[8] He confirmed Emmerling's[9] observation and showed that cystine is a normal cleavage product of keratins. The same observation was also made independently, about the same time, by Embden[1] in Hofmeister's laboratory, who isolated cystine from the cleavage products of egg-albumin, serum-albumin and edestin.

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[1] *Zeitschr. f. Biol.*, xxvii, p. 415.

[2] *Chem. Zeit.*, 1894, No. 80. *Verh. Ges. deutsch. Naturf. u. Aertzte*, ii, p. 391, 1894.

[3] *Zentrbl. f. Physiol.*, x, p. 529.

[4] *Zeitschr. f. physiol. Chem.*, xx, p. 253.

[5] Dissertation, Marburg, 1898.

[6] *Journ. f. prakt. Chem.*, (2) lvii, p. 365, 1898.

[7] *Maly's Jahresbericht*, xxiii, p. 43.

[8] *Zeitschr. f. physiol. Chem.*, xxviii, p. 595.

[9] Emmerling: *loc. cit.*

This work of Mörner and Embden was followed by the important researches of Friedmann[2] in 1902, on the constitution of cystëine and the physiologically important mercapturic acids. Baumann[3] had already established the chemical relationship between cystine and cystëine and reviewed the former as the disulphide of α, α-mercaptoaminopropionic acid (cystëine). Friedmann proved, however, that Baumann's structural formulae were incorrect and showed that cystëine is α-amino-β-mercaptopropionic acid, and consequently that cystine is an α, β-derivative and not an α,α compound as assumed by Baumann. Friedmann[4] also showed that α-mercaptopropionic acid, which had previously been detected by Suter[5] is also an important cleavage product of keratins. He separated it, in the form of its benzyl derivative, from the acid solutions after hydrolysis of wool, horn, feathers and hair.

A new view point and direction was given to the question of loosely bound sulphur in proteins by this work of Mörner, Embden and Friedmann. Since it was known that proteins and cystine are decomposed by alkali, under similar conditions, with formation of alkali sulphide, the question was raised whether the sulphur linkage represented in this sulphur cleavage product is the only one to be dealt with in proteins.

Mörner[6] attempted to answer this question. He observed that about the same percentage of sulphur (75 per cent) was detached from cystine by heating with alkali, as was obtained by heating horn, hair, serum globulin and serum albumin under the same conditions. He also found in the solutions from which cystine had been separated a quantity of sulphur, which yielded about the same proportion of lead sulphide. This soluble sulphur compound however was not identified. Because of this constant proportion of detachable sulphur Mörner therefore assumed that this element was combined only in the form of cystine. Basing his calculations on this assumption he calculated quantities of cystine, which accounted for nearly all the sulphur represented in the above sulphur proteins. On the other hand, Mörner concluded that the membranes of eggs, ovalbumin and fibrinogen contain other sulphur bodies than cystine. For example, he could account for only a small proportion of the sulphur as cystine in ovalbumin, and actually observed the volatilization of a sulphur compound. This was not identified and represented about one-third of the total sulphur content of this protein. He writes as follows: "Da es möglich ist etwa ein Drittel des Schwefels in der Form einer fluchtigen Substanzen zu erhalten, ist es nicht unwahrscheinlich dass der Schwefel dieses Eiweiss in drei verschiedenen Formen gebunden ist."

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[1] *Zeitschr. f. physiol. Chem.*, xxxii, p. 94.

[2] *Hofmeister's Beiträge*, ii, p. 433; iii, p. 1. Neuberg: *Ber. d. chem. Ges.*, xxxv, p. 3161.

[3] *Zeitschr. f. physiol. Chem.*, viii, p. 299, 1884.

[4] *Hofmeister's Beiträge*, iii, 184, 1903.

[5] *Zeitschr. f. physiol. Chem.*, xx, p. 577, 1895.

[6] *Ibid.*, xxxiv, p. 207.

Notwithstanding the fact that Mörner's method of calculation was undoubtedly very uncertain, nevertheless, physiological chemists attach much importance to his work and still lay great stress on the fact that cystine behaves as a protein when heated with alkali, and consequently is the source of the loosely bound sulphur in these natural substances. The attention has been focused practically wholly on the cystine molecule and no careful consideration given to the possibility that there may be other unstable sulphur groupings in some proteins, which can break down, on hydrolysis, giving hydrogen sulphide. The fact that cystine behaves like a sulphur protein when heated with alkali, or that other primary sulphur cleavage products have not been isolated, does not preclude the possibility of there being other sulphur combinations, which can disintegrate to give hydrogen sulphide. This assumption is worthy of some consideration and cannot be excluded from the discussion of this important question. It is not inconceivable that in the digestion of proteins with alkalies and acids, there may be atomic displacements involving the removal of unsaturated sulphur, as in $C = S$, which can even occur before the cleavage of the protein itself and the liberation of the cystine molecule.

Wohlgemuth,[1] who views cystine as the principal, if not the only mother substance of the sulphates, the unoxidized sulphur and the salts of dithionic acid, $H_2S_2O_6$, in the urine; of taurine in the bile and the sulphur products of intestinal decomposition, viz: hydrogen sulphide, methyl mercaptan and ethyl sulphide, writes as follows: "Sollte sich in Zukunft heraustellen, dass neben dem Cystin noch andre schwefelhaltige Bestandteile im Eiweiss enthalten sind, so dürften dieselben für den Stoffwechsel in der angegebenen Richtung von untergeordneten Bedeutung sein."

Much evidence can be adduced in support of the assumption that other groupings besides cystine are present in some proteins, which break down on hydrolysis giving hydrogen sulphide. It is well known for example that the ratio between sulphur split off by alkali and that firmly bound differs decidedly in the various proteins so far examined. This is well brought out by examination of the percentages of loosely bound sulphur recorded in the following table, which have been abstracted from a paper on "Sulphur in Proteins" by Osborne.[2] Whether these remarkable differences in amounts of loosely bound sulphur are to be explained by the different proportions of cystine in these proteins or by varying percentages of different unstable sulphur groupings, we have not as yet convincing evidence. An interesting feature of this work is the fact that the percentages of sulphur detached by boiling with 30 per cent sodium hydroxide and lead acetate were not increased by heating the proteins for the same length of time at 165°.

Suter[3] in Baumann's laboratory compared the velocity of lead sulphide formation from different proteins with that of sulphide formation from pure cystine. By using alkali of the same strength and working otherwise under practically the same conditions he observed that 60 per cent of the sulphur in cystine was split off after nine hours boiling with alkali, while hair lost under the same conditions 98.8 per cent and feathers 98.0 per cent of their sulphur after fifteen hours heating.

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[1] *Zeitschr. f. physiol. Chem.*, xl, p. 81; xliii, p. 469.

[2] *Report of Conn. Agr. Exp. Sta.*, 1900, p. 443.

[3] *Zeitschr. f. physiol. Chem.*, xx, p. 564, 1895.

TABLE I.

Ratio Between Loosely Bound Sulphur and Total Sulphur.

	PER CENT OF LOOSELY BOUND SULPHUR.
Seralbumin	66.0
Oxyhaemoglobin (dog)	59.0
Serglobulin (horse)	57.0
Gliadin	60.0
Oxyhaemoglobin (horse)	50.0
Vignin	50.0
Amandin	50.0
Globin	48.0
Glycinin	46.0
Vicilin	46.0
Legumin	41.0
Edestin	40.0
Zein	35.0
Ovovitellin	34.0
Fibrin	34.0
Excelsin	32.0
Ovalbumin	30.0
Phaseolin	23.0
Casein	13.0

Pick[1] made the very interesting observation that the albumoses prepared from fibrin, which contains about 1.2 per cent of cystine[2] gave off by treatment with alkali the whole of their sulphur in the form of hydrogen sulphide. He concluded therefore that the sulphur in these proteins cannot be present as cystine. His figures are given in the following table:

TABLE II.

	HETEROALBUMOSE	PROTALBUMOSE
	per cent	*per cent*
Total sulphur	1.27	1.30
Sulphur removed by alkali	1.14	1.24

Hofmann and Pregl[3] observed that *koilin* gives lead sulphide when digested with alkali and lead acetate, but were unable to

[1] *Zeitschr. f. physiol. Chem.*, xxviii, p. 254, 1899.
[2] Mörner: *Ibid.*, xxxiv, p. 207.
[3] *Ibid.*, lii, p. 465.

isolate cystine from the products of hydrolysis. The following statement is abstracted from their paper:

> —"Die Unmöglichkeit, aus der in Arbeit genommenen Koilinmenge Cystin in Substanz zu isolieren insbesondere aber das Fehlen das Schwefelbleireaktion an der aus der ammoniakalischen Lösing erhaltenen Krystallisation, lässt die Frage unentschieden ob die nach dem Verfahren von Mörner zum Zwecke der Berechnung der Cystinmenge gefundenen Schwefelwerte wirklich auf Cystin zu beziehen sind oder nicht." And also as follows:—"anderseits ist aber die Möglichkeit auch nicht ausser acht zu lassen, dass letztere Reaktion (Schwefelbleireaktion) sowie der nach dem Mörnerschen Verfahren bestimmte Schwefel auf einen Baustein im Koilin zu beziehen ist, der mit dem Cystin nicht identisch ist."

Buchtala[1] determined the relative proportions of cystine formed by hydrolysis of men's hair and nails, horses' hoofs, cattle-hair and hoofs, and swine's hoofs and bristles with strong hydrochloric acid. He obtained the largest yield of cystine from men's hair, and also made the important observation that sulphur deposited in his condenser tubes during all his hydrolyses except in the case of horses' hoofs. He states that no sulphur separated during the digestion of this material and makes the following statement regarding the behavior of swine's hoofs and bristles under the same conditions:—"Schwefel schied sich in betrachtlicher Menge ab." He also observed later[2] that no sulphur separated during the hydrolysis of hens' claws with concentrated hydrochloric acid, while on the other hand, this element separated during the hydrolysis of horn shavings under the same conditions. Apparently physiological chemists have paid little attention to this property of proteins depositing sulphur on hydrolysis. It is not improbable that there is some connection between this property and the nature of the sulphur linkages in these substances.

Cohn,[3] who hydrolyzed horn shavings with hydrochloric acid for five hours, writes as follows:—"Die entweichenden Gase rochen nach Schwefelwasserstoff aber nach Mercaptan ahnlich." Buchtala[4] subjected to hydrolysis with concentrated hydrochloric acid the egg membranes of three different species of sharks, viz.: *Scyllium stellare* (1.44 per cent S), *Pristiurus melanostonus* (1.52 per cent S) and *Scyllium canicula* (1.33 per cent S) —but after repeated trials he did not succeed in isolating any cystine. He states that it is questionable whether any sulphur is bound in these keratins as cystine.

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[1] *Zeitschr. f. physiol. Chem.*, lii. p. 474, 1907.
[2] *Ibid.*, lxix, p. 210.
[3] *Ibid.*, xxvii, p. 410.
[4] *Ibid.*, lvi. p. 11. 1908.

Rettger[1] observed that milk proteins undergo partial decomposition on heating normal milk above 85 degrees C. This was indicated by the liberation of a volatile sulphide, in all probability hydrogen sulphide, which was easily recognized by the blackening of lead acetate paper and the decolorization of a potassium permanganate solution.

Bauditsch[2] observed that when wool was allowed to stand at ordinary temperature suspended in neutral hydrogen peroxide solution, the liquid finally became acid due to the formation of sulphuric acid. Chlorinated wool, on the other hand, gave no sulphuric acid under the same conditions. He therefore concluded that at least a part of the sulphur in proteins is bound to oxygen.

Raikow[3] allowed phosphoric acid to act on unbleached wool and men's hair at ordinary temperature and observed that sulphur dioxide was evolved. Since sulfonic acids and sulphates do not react under the same conditions giving sulphur dioxide, Raikow therefore concluded that the sulphur is partly linked in keratins in sulphite form. Grandmougin[4] on the other hand, claims that bleached wool does not give off sulphur dioxide when subjected to the action of phosphoric acid.

To sum up, it therefore appears very probable from a consideration of the above evidence that there are other sulphur combinations[5] in proteins besides the cystine group, which can break down, on hydrolysis, with formation of hydrogen sulphide. It seemed very desirable therefore to study some types of sulphur groupings, which have not been considered and which may be present in these natural substances. No attempt has been made, so far as the writer is aware, to determine by synthetical methods the nature of such possible sulphur linkages. In order therefore to obtain new data, which may possibly help to advance our present knowledge of this important question of loosely bound sulphur, and consequently help to arrive at a clearer understanding of the various normal and pathological changes of this element in animal and plant organisms, we have begun a systematic study of the thioamides of amino-acids. The following paper is the first contribution to this investigation.

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[1] *Amer. Journ. of Physiol.*, vi, p. 450, 1902.

[2] *Chem. Zeit.*, xxxii, p. 620.

[3] *Chem. Zeit.*, xxix, p. 900; *Chem. Zentrbl.*, 1905, ii, p. 970.

[4] *Chem. Zeit.*, xxxi, p. 174; *Chem. Zentrbl.*, 1907, i, p. 1604.

[5] Osborne and Guest: This *Journal*, ix, p. 333.

THIOAMIDES: THE FORMATION OF THIOPOLYPEPTIDE DERIVATIVES BY THE ACTION OF HYDROGEN SULPHIDE ON AMINOACETONITRILE.

By TREAT B. JOHNSON and GERALD BURNHAM.

(*From the Sheffield Laboratory of Yale University.*)

(Received for publication, May 22, 1911.)

The two types of oxygen groupings represented among the various cleavage products of proteins are the hydroxyl group OH, which is present in the amino-acids, serine (I), oxyproline (III) and tyrosine (V), and the keto form, –COOH and CONH–, present in the polypeptides (VII) and their anhydrides (IX), in asparagine and the amino-acids themselves. If one takes a practical, chemical view of sulphur proteins and considers them as oxygen proteins in which oxygen has been partially displaced by bivalent sulphur, then theoretically only two types of sulphur combinations need to be taken into consideration viz: mercapto SH and thioamide, CSNH or C(:NH)SH, corresponding to the two oxygen groupings. Cystëine (II) represents a sulphur cleavage product corresponding to the oxygen acid serine (I). The corresponding sulphur derivatives of oxyproline and tyrosine, viz: *thioproline* (IV) and *thiotyrosine* (VI) have not been detected among the cleavage products of proteins or prepared synthetically.

The sulphur analogues of the polypeptides and their anhydrides (diacipiperazines) viz.: *thiopolypeptides* (VIII) and *dithiopiperazines* (X), represent two new classes of organic compounds. A knowledge of their chemical properties is therefore of the greatest importance, because of the possibility that thiopolypeptide groupings –$NHCH_2CS$– may be present in some sulphur proteins.[1]

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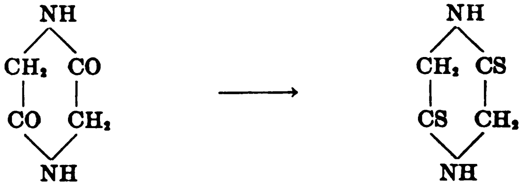
[1] Preliminary paper, This *Journal*, ix, p. 331, 1911.

$$\begin{array}{ccc} HOCH_2CH(NH_2)COOH & \rightarrow & HSCH_2CH(NH_2)COOH. \\ \text{I} & & \text{II} \\ \text{(Serine)} & & \text{(Cystëine)} \end{array}$$

$$\begin{array}{ccc} C_3H_6N.CH(OH)COOH & \rightarrow & C_3H_6N.CH(SH)COOH \\ \text{III} & & \text{IV} \\ \text{(Oxyproline)} & & \text{(Thioproline)} \end{array}$$

$$\begin{array}{ccc} HOC_6H_4CH_2CH(NH_2)COOH & \rightarrow & HSC_6H_4CH_2CH(NH_2)COOH \\ \text{V} & & \text{VI} \\ \text{(Tyrosine)} & & \text{(Thiotyrosine)} \end{array}$$

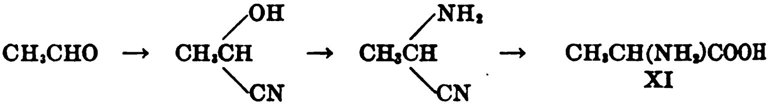
$$\begin{array}{ccc} NH_2CH_2CONHCH_2COOH & \rightarrow & NH_2CH_2CSNHCH_2COOH \\ \text{VII} & & \text{VIII} \\ \text{(Glycylglycine)} & & \text{(Thioglycylglycine)} \end{array}$$



IX X

(Diacipiperazine) (Dithiopiperazine)

In 1850, Strecker[1] published his important paper describing the synthesis of α-alanine (XI) from acetaldehyde, ammonia and hydrocyanic acid. Thirty-eight years later, in 1888, Weyl[2] discovered this amino-acid among the cleavage products of fibroin. Strecker's method of synthesizing α-amino-acids has not only received a wide application, but it has also assumed great biological importance owing to the fact that formaldehyde, ammonia and hydrocyanic acid are believed to be primary assimilation products in the metabolism of proteins.



$$CH_3CHO \rightarrow CH_3CH\begin{cases}OH\\CN\end{cases} \rightarrow CH_3CH\begin{cases}NH_2\\CN\end{cases} \rightarrow \underset{\text{XI}}{CH_3CH(NH_2)COOH}$$

Treub,[3] for example, assumes that prussic acid, formed from sugar and inorganic nitrates, is one of the first assimilation products·

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[1] *Ann. Chem. (Liebig)*, lxxv, p. 28.

[2] *Ber. d. chem. Ges.*, xxi, p. 1529.

[3] *Ann. Jard. bot. Buitengorg*, 1895, 1904.

nts and that this unites with formaldehyde and ammonia
g amino nitriles,which are then hydrolyzed to the amino-acids.
en[1] has suggested that the polypeptides may be formed in a
r manner by the interaction of acid amides and the nitriles
e α-hydroxy-acids.

ow these amino nitriles play, as is assumed, so important
in the formation of proteins from simpler substances, then
very desirable to have knowledge of their behavior towards
gen sulphide. So far as the writer is aware the action of this
nt on nitriles of this character has not been investigated. It
plausible that hydrogen sulphide may be the first assimila-
product of inorganic sulphates ($CaSO_4$,$MgSO_4$) and that thio-
groupings functionate in the synthesis of sulphur proteins, be-
rmed by the addition of hydrogen sulphide to the amino nitriles.
important investigations of Beijerinck[2], Saltet[3] and van
n[4] lend support to such an assumption. These investigators
isolated pure cultures of anaërobic bacteria from brackish
s and swamp mud, which are capable of reducing inorganic
ates directly to hydrogen sulphide and sulphur.

have now examined the action of hydrogen sulphide on the
est aminonitrile of the above type viz.: aminoacetonitrile,[5]
CH_2CN, which is formed by the action of ammonia and hydro-
c acid on formaldehyde, and have obtained some very inter-
results. This nitrile reacts normally with hydrogen sulphide
dinary temperature, giving the unknown thioamide of gly-
(XII). This amide however was not isolated. It is ex-

$$NH_2\,CH_2CN + H_2S = \underset{\text{XII}}{NH_2CH_2CSNH_2}$$

ly unstable and slowly undergoes, in alcoholic solution, a
aneous decomposition giving the thiopolypeptide derivative
glycylglycinthioamide (XIII) with loss of a molecule of am-
. This change is represented by the following equation

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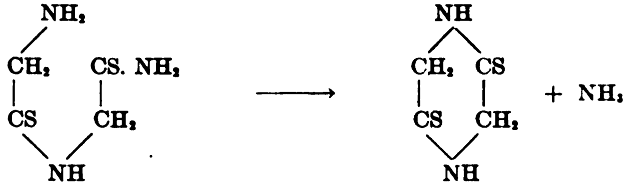
zungsber. Heidelberger Akad. Wiss., 1910.
ntrbl. f. Bakt., 1895, ii, p. 1.
d., 1900, ii, p. 648.
ntrbl. f. Bakt., 1903, ii, pp. 81, 113; also Goslings: *Ibid*, 1904, ii, p. 385.
ages. *Ber. d. chem. Ges.*, xxxvi, p. 1514.

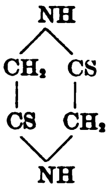
$$NH_2CH_2CSNH_2 + NH_2CH_2CSNH_2 = \underset{XIII}{NH_2CH_2CS.NHCH_2CSNH_2} + NH_3.$$

This thioamide is the sulphur analogue of glycylglycinamide $NH_2CH_2CONHCH_2CONH_2$, whose carbonyl and carbethoxy derivatives have been described by Fischer.[1] Its structure was proved by the facts that it dissolved in both acids and bases, and gave on hydrolysis with hydrochloric acid glycocoll hydrochloride, hydrogen sulphide and ammonium chloride,

$$NH_2CH_2CSNHCH_2CSNH_2 + 4H_2O + 3HCl = 2NH_2CH_2COOH.HCl + NH_4Cl + 2H_2S.$$

Thioglycylglycinthioamide (XIII) is not the only product formed by interaction of hydrogen sulphide with aminoacetonitrile. This thioamide undergoes an inner condensation, at ordinary temperature, giving the hitherto unknown dithiopiperazine (XIV) with loss of a molecule of ammonia. In fact this cyclic derivative is the chief product of the reaction. The structure of this piperazine is established by the following facts: It dissolves in cold alkali and is precipitated unaltered by addition of hydrochloric acid. It possesses no basic properties proving the absence of a free amino group, and undergoes hydrolysis with strong hydrochloric acid giving glycocoll hydrochloride and hydrogen sulphide. There was no evidence of the formation of any ammonium chloride.


XIII XIV



$$+ 4H_2O + 2HCl = 2NH_2CH_2COOH \cdot HCl + 2H_2S$$

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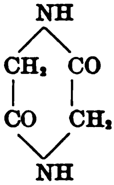
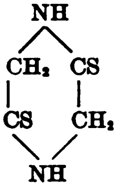
[1] *Ber. d. chem. Ges.*, xxxv, p. 1095.

The relationship between aminoacetthioamide and dithiopiperızine is therefore similar to that between ethyl aminoacetate and liacipiperazine. Curtius,[1] for example, has shown that this ester ikewise undergoes a spontaneous decomposition in aqueous solu:ion, at ordinary temperature, giving diaciperazine corresponding ;o dithiopiperazine. He also observed that this piperazine was ıot the only product of this decomposition, but isolated another ıubstance (biuretbase) which he proved to be the ethyl ester of :riglycylglycin (XV). These relationships are expressed by the 'ollowing formulas. It is of interest to note here that we have

$$NH_2CH_2COOC_2H_5 \rightarrow \underset{XV}{NH_2CH_2CO(NHCH_2CO)_2NHCH_2COOC_2H_5} \rightarrow$$



$$NH_2CH_2CSNH_2 \rightarrow NH_2CH_2CS.NHCH_2CSNH_2 \rightarrow$$



'easons for believing that trithioglycylglycinthioamide NH_2CH_2CS-$(NHCH_2CS)_2$ $NHCH_2CSNH_2$ corresponding to the ester (XV), is ılso formed by the decomposition of aminoacetthioamide, but were unable to isolate it sufficiently pure for analysis.

This property of a thioamide reacting as an ester with bases and giving a new thioamide with loss of ammonia is not a common reaction for thioamides. The reaction in the case of aminoıcetthioamide is unique, because it takes place at such low temperıtures. Analogous transformations have been described by

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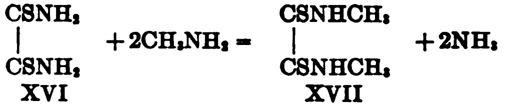
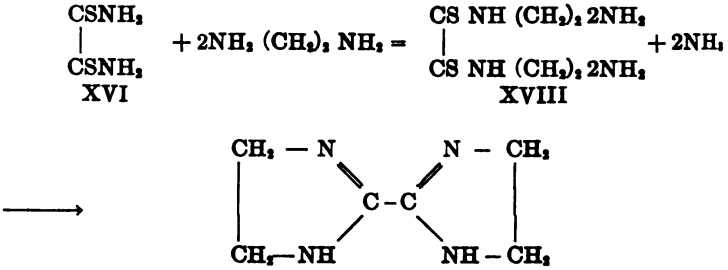
[1] Curtius and Göebel: *Journ. f. prakt. Chem.*, (2). xxxvii, p. 170; Cur:ius: *Ber. d. chem. Ges.*, xxxvii, p. 1284.

Wallach[1] who observed, for example, that amino groups in dithiooxamide (rubeanwasserstoff) can be displaced by heating with primary aliphatic amines giving substituted thioamides (XVII). Aromatic bases however did not react with the thioamide.



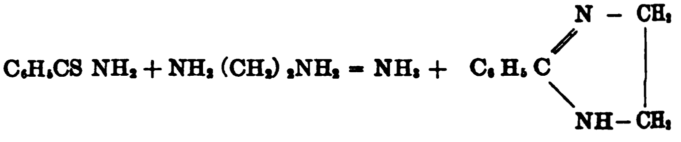
$$\begin{matrix}CSNH_2\\|\\CSNH_2\\XVI\end{matrix} + 2CH_3NH_2 = \begin{matrix}CSNHCH_3\\|\\CSNHCH_3\\XVII\end{matrix} + 2NH_3$$

Forster and Forsell[2] also heated dithiooxamide with ethylenediamine and obtained bisdihydroglyoxalin (XIX). This transformation involved the intermediate formation of the aminothioamide (XVIII). Ethylene diamine and thiobenzamide reacted in a similar manner, on heating, giving phenyldihydroglyoxalin (XX).



$$\begin{matrix}CSNH_2\\|\\CSNH_2\\XVI\end{matrix} + 2NH_2(CH_2)_2NH_2 = \begin{matrix}CS\,NH\,(CH_2)_2NH_2\\|\\CS\,NH\,(CH_2)_2NH_2\\XVIII\end{matrix} + 2NH_3$$

XIX



$$C_6H_5CS\,NH_2 + NH_2(CH_2)_2NH_2 = NH_3 + \ldots$$

XX

The study of thioamides of amino-acids will be continued in this laboratory. Since it is known that the oxygen amides can be obtained by disulphurization of thioamides, this synthesis of thiopolypetide derivatives betokens a new method of preparing polypeptide compounds.

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[1] *Ann. Chem.* (*Liebig*), cclxii, p. 354.

[2] *Ber. d. chem. Ges.*, xxv, p. 2132.

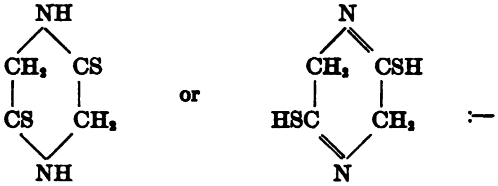
EXPERIMENTAL PART.

Aminoacetonitrile Sulphate, $NH_2CH_2CN.H_2SO_4$.

This sulphate, which was used in this investigation, was prepared according to Klage's[1] directions by hydrolysis of methyleneaminoacetonitrile $(CH_2:N.CH_2CN)_x$ with dilute sulphuric acid.

Action of Hydrogen Sulphide on Aminoacetonitrile.

Dithiopiperazine.



Different products are formed by the action of hydrogen sulphide on aminoacetonitrile, in alcohol solution, depending upon the temperature and length of time of treatment with the gas. It was very difficult therefore to regulate the conditions so as to obtain this piperazine in good yields and free from impurities. The products of the reaction were always contaminated with dark-colored, melanin-like substances if the hydrogen sulphide was allowed to act on the nitrile above 50–60.°

Experiment I. Twenty grams of aminoacetonitrile sulphate were finely pulverized and suspended in 100 cc. of alcohol, which had been saturated with ammonia gas at 0.° On conducting hydrogen sulphide gas into the solution at 0° it assumes a light yellow color, but if the solution is allowed to become warm during this operation it takes on a dark red color and secondary products are formed. After continual treatment with the gas for about 4 hours the above piperazine had separated as a light brown sandy powder mixed with ammonium sulphate. This was filtered off and the alcoholic filtrate saved (see below). The thiopiperazine was separated from ammonium sulphate by trituration with cold water and then dried at 100.° The weight was 3.5 grams. This compound is insoluble in boiling water and alcohol. It is a weak acid and dissolves in cold, dilute sodium hydroxide solution, without decomposition, giving a red solution of its disodium salt. It is reprecipitated unaltered from solutions of its sodium salt by addition of hydrochloric or sulphuric acids as a light grey microscopic powder. The compound is decomposed by digestion in sodium hydroxide solution with formation of sodium sulphide and the sodium salt of glycocoll. There was no evidence of any evolution of ammonia proving the absence of thioamide nitrogen—$CSNH_2$. It has no definite melting or decomposition point, but begins to turn brown at about 160° and at 270–280° becomes jet black, but does not effervesce below 300.° In order to purify the compound it was always necessary to dissolve it in cold alkali and reprecipitate it by addition of acids. An explanation for this procedure will be given below. The lead and mercury salts of the piperazine separate at once on addition of lead acetate and mercury chloride respectively to an aqueous solution of the sodium salt. They are both light-brown colored salts, which are apparently quite stable at ordinary temperature but decompose on heating in a water bath giving lead and mercury sulphides. The silver salt separates in a gelatinous condition and begins to decompose at once with formation of black silver sulphide.

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[1] *Loc. cit.*

Analyses: I. 0.1942 gram gave NH_3 = 0.03693 gm N.
II. 0.1405 gram gave NH_3 = 0.0268 gm. N.

	Found:		Calculated for $C_2H_6N_2S$:	Calculated for $C_4H_6N_2S_2$:
N.............	I. 19.02	II. 19.07	30.1	19.20

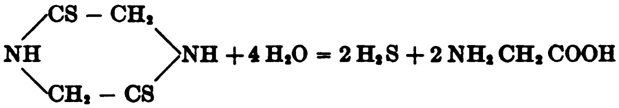
After filtering from the thiopiperazine the ammoniacal filtrate (above) was allowed to evaporate spontaneously at ordinary temperature when a yellow colored, amorphous substance finally deposited. After the alcohol had completely evaporated however this substance gradually assumed a red color, indicating oxidation or a secondary change, and a gummy residue adhered to the sides of the dish. These transformations on exposure to the atmosphere and especially on heating were very remarkable. The material was first triturated with cold water to remove any ammonium sulphate present, and then, after drying, triturated with carbon bisulphide to remove any sulphur. The product thus obtained was a dark brown powder and the weight was 2.0 grams. When heated in a capillary tube it began to yield at 70,° turned dark colored and then melted from 89–95° with evolution of hydrogen sulphide and forming a black tar. This substance was insoluble in 95 per cent alcohol and cold water. When warmed with water it slowly underwent decomposition with evolution of hydrogen sulphide and a clear solution was finally obtained. It dissolved in cold, dilute sodium hydroxide solution giving a red solution. On adding hydrochloric acid a brown precipitate separated in neutral solution but dissolved again on adding an excess of acid, showing both basic and acid properties. When the sodium hydroxide solution was heated ammonia was immediately evolved and then on acidifying the solution with hydrochloric acid hydrogen sulphide was copiously evolved indicating the presence of sodium sulphide in solution. The compound dissolved in dilute hydrochloric acid giving a clear solution which evolved hydrogen sulphide on warming. The chemical behavior of the substance indicated the thioamide of aminoacetic acid—$NH_2CH_2CSNH_2$. A nitrogen determination however proved that it was not this amide but a *thiopolypeptide* derivative corresponding to glycylglycinamide—$NH_2CH_2CONHCH_2CONH_2$— or *thioglycylglycinthioamide,* $NH_2CH_2CSNHCH_2CSNH_2$:

Analyses: 0.1576 gram gave NH_3 = 0.03875 gram N.

	Found:	Calculated for $C_4H_9N_3S_2$:	Calculated for $C_2H_6N_2S$:
N	24.6	25.8	30.1

The low result obtained for nitrogen was due to the presence of a small amount of the thiopiperazine as impurity. This thiopolypeptide derivative always deposits with the above piperazine when conducting hydrogen sulphide into the alcoholic solution of the aminoacetonitrile. Therefore the necessity of purifying the cyclic compound for analysis, as described above, by dissolving it in cold alkali and reprecipitating with an excess of acid. The basic thioglycylglycinthioamide is dissolved by the excess of acid. All our attempts to isolate from the ammoniacal solutions the plain thioamide —$NH_2CH_2CSNH_2$— were unsuccessful. It seems not improbable that tri- and even tetrathiopolypeptides may also have been formed here as well as the thioamide which we isolated. The residues obtained after spontaneous evaporation of our ammoniacal solutions contained sulphur and were strongly basic indicating the presence of such combinations, but we were unable to isolate a substance from them sufficiently pure for a reliable analysis.

Hydrolysis of Dithiopiperazine with Hydrochloric Acid.



One gram of the dithiopiperazine was suspended in 20 cc. of concentrated hydrochloric acid, in a flask connected with a return condenser, and then heated on the steambath. The piperazine dissolved very slowly with evolution of hydrogen sulphide and after one hour's digestion there was still some unaltered material, which had not dissolved. The solution was then heated for another hour when a clear red solution was obtained with a small amount of free sulphur in suspension. A thin film of white sulphur had also deposited in the condenser tube. After filtering, the solution was evaporated to dryness when we obtained a crystalline substance colored a deep red and mixed with a small amount of dark colored amorphous material. The residue was dissolved in boiling water, decolorized with bone-black and then allowed to evaporate in a vacuum over sulphuric acid. We thus obtained a colorless, crystalline deposit of the pure hydrochloride of aminoacetic acid. When some of the salt was dissolved in dilute sodium hydroxide solution and this was boiled, there was no evolution of ammonia proving the absence of any ammonium chloride. The hydrochloride was dried for analysis at 100.°

Analyses I. 0.2387 gram gave NH_3 = 0.03110 gram N.
II. 0.2505 gram gave NH_3 = 0.03250 gram N.

	Found:		Calculated for $C_2H_5O_2N$. HCl:
N	I. 12.9	II. 13.0	12.6

Some of the hydrochloride was dissolved in cold water and Baumann and Schotten's reaction applied with benzoylchloride and sodium hydroxide. No crystalline material separated from the alkaline solution proving the absence of benzamide and consequently the absence of ammonium chloride in the glycine salt. When the alkaline solution was acidified with hydrochloric acid hippuric acid separated in prismatic crystals and melted after one crystallization from dilute alcohol at 186–187.° A mixture of this substance and some pure hippuric acid melted at the same temperature.

Hydrolysis of Thioglycylglycinthioamide with Hydrochloric Acid:

$$NH_2CH_2CSNHCH_2CSNH_2 + 4H_2O = 2NH_2CH_2COOH + 2H_2S + NH_3.$$

One gram of the thioamide was dissolved in 20 cc. of concentrated hydrochloric acid and the solution boiled for two hours. Hydrogen sulphide was evolved and sulphur deposited in the condenser tube. The dark red solution was filtered and evaporated on the steam bath when we obtained a thick, gummy substance, which was very soluble in water and still contained sulphur. This was dissolved again in a large volume of concentrated hydrochloric acid and the solution allowed to evaporate on the steam bath. We then obtained a dark colored viscous residue, which dissolved immediately in cold water. Dilute sodium hydroxide was then added until the solution was neutral when a slight amorphous precipitate separated. This was filtered off, the solution acidified with hydrochloric acid and then decolorized by long digestion with bone-coal. This clarified solution continually assumed a dark red color on evaporating in the air, and in order to prevent this change it was necessary to evaporate in a vacuum, at ordinary temperature, over sulphuric acid. We thus obtained a crystalline deposit of the hydrochloride of amino-acetic acid mixed with ammonium and sodium chlorides. The presence of ammonium chloride was proved by the fact that ammonia was copiously evolved immediately on warming some of the material with sodium hydroxide. Since it was impossible to separate the hydrochloride of glycocoll by crystallization, its presence was established by dissolving the mixed salts in water and applying a Baumann and Schotten reaction with benzoylchloride. On acidifying the solution a mixture of benzoic and hippuric acids was obtained. The benzoic acid was first removed from this mixture by digestion with ligröin and the insoluble hippuric acid then recrystallized from dilute alcohol. It separated in prisms melting at 185–187°. A mixture of this material and pure hippuric acid melted at the same temperature.

Experiment II. In this experiment 50 grams of the sulphate of amino-acetonitrile were finely pulverized and then sifted into 150 cc. of cold, concentrated alcoholic ammonia. After thorough agitation in order to obtain a complete decomposition of the salt the undissolved ammonium sulphate was then separated by filtration. Hydrogen sulphide gas was conducted into the ammoniacal solution of the free nitrile, at 0°, for exactly one hour. The solution was still clear and no piperazine had separated. It was then allowed to stand, at ordinary temperature, for 10 hours before being examined again. The alcohol had then assumed a light yellow color and a light grey powder had deposited on the bottom and sides of the flask. This substance did not melt below 300° and the weight was 5.0 grams (Crop I). Hydrogen sulphide was again conducted into the solution for one hour at 0° and then allowed to stand again for about 10 hours when we obtained 4.5 grams more of a fine powder which did not melt or effervesce below 300° (Crop II). The alcoholic filtrate was then saturated with hydrogen sulphide gas for one hour and allowed to stand again at ordinary temperature when more material continued to deposit. The alcohol solution was finally concentrated at ordinary temperature under diminished pressure to a volume of 20–25 cc. and the undissolved material separated. This weighed 3.0 grams and did not melt below 300°. On further evaporation of the alcohol filtrate a black melanin-residue containing sulphur was obtained from which we did not succeed in isolating a definite substance. The 3.0 grams of material melting above 300° were washed with water to remove all traces of ammonium sulphate and then dried for analysis over sulphuric acid. The nitrogen determinations indicated that we were dealing with dithiopiperazine contaminated with a little thioglycylglycinthioamide or the amide of glycocoll. That this

***Analysis*:** I. 0.3088 gram gave NH_3 = 0.06199 gram N.
II. 0.1465 gram gave NH_3 = 0.02962 gram N.

	Found:		Calculated for $C_4H_6N_2S_2$:	Calculated for $C_4H_9N_3S_2$:
N	I. 20.0	II. 20.2	19.20	25.8

assumption was correct was shown by the behavior of the material towards sodium hydroxide solution. It dissolved in dilute alkali with slight evolution of ammonia, and on acidifying the solution with dilute hydrochloric acid hydrogen sulphide was evolved and the dithiopiperazine deposited as an insoluble, light-grey powder, which did not decompose or melt below 300°.

Examination of Crop I (Above).

Analyses: 0.1691 gram gave NH_3 = 0.03403 gram. N.

	Found:	Calculated for $C_4H_6N_2S_2$:
N	20.13	19.20

This crop of 4.8 grams was dissolved in 25 cc. of a cold sodium hydroxide solution when ammonia was evolved and a clear solution resulted. After filtering from a trace of sulphur the solution was diluted with water to 200 cc. and then acidified with hydrochloric acid. Hydrogen sulphide was evolved and 3.9 grams of the dithiopiperazine separated and on analysis gave 19.00 per cent nitrogen. In order to prove the presence of thioglycylglycinthioamide in the acid filtrate, it was diluted with concentrated hydrochloric acid and then evaporated to dryness on the steam bath. We obtained a dark colored crystalline residue which dissolved in cold water with the exception of a trace of sulphur. This solution was made alkaline with sodium hydroxide, when ammonia was evolved, and then shaken with a few drops of benzoyl chloride. On acidifiying the solution and cooling a mixture of benzoic and hippuric acids deposited. The benzoic acid was removed by digestion with ligröin, and the insoluble material purified by crystallization from dilute alcohol. It melted at 187° and when mixed with hippuric acid the melting point was not changed. Therefore the above filtrate contained thioglycylglycinthioamide which underwent hydrolysis by heating its acid solution giving aminoacetic acid, ammonia and hydrogen sulphide.

Examination of Crop II (Above).

Analyses: I. 0.3776 gram gave NH_3 = 0.0815 gram N.
II. 0.2713 gram gave NH_3 = 0.05869 gram N.

	Found		Calculated for $C_4H_6N_2S_2$:	Calculated for $C_4H_9N_3S_2$:
N	I. 21.5	II. 21.6	19.20	25.8

This crop similar to crop I, was apparently a mixture of the dithiopiperazine and thioglycylglycinthioamide. In order to separate the piperazine the material was dissolved in sodium hydroxide and then precipitated in the usual way with acid. There was a strong evolution of hydrogen sulphide and 1.6 grams of the piperazine separated as a light grey powder.

THE DIGESTIBILITY OF WHITE OF EGG AS INFLUENCED BY THE TEMPERATURE AT WHICH IT IS COAGULATED.

By PHILIP FRANK.

(From the Physiological Laboratories of the Tulane University of Louisiana, New Orleans.)

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In the course of an investigation in which the Mett tube method was used for standardizing ferments, it was suggested by Prof. Gustav Mann, that it would be interesting to determine whether the digestibility of egg albumin could be affected by the temperature at which it had been coagulated. For, although egg albumin coagulates at 73° C. (Halliburton), yet the presence of other constituents, particularly globulin, in the white of egg might so affect the state of coagulation of the egg albumin proper as to show a difference in the rate of digestion between white of egg gradually heated to the point of coagulation of the egg albumin and that suddenly coagulated at higher than the coagulating temperature.

Accordingly, the whites of fresh eggs were beaten into a stiff foam and then allowed to stand in the ice-chest over night. From the liquid that separated out, Mett tubes were made as follows: A glass tube of about 2 mm. bore and of about 30 cm. length was bent upwards and outwards at both ends, to allow of its suspension in a horizontal plane during coagulation, and the egg-white was sucked up into it. A large pan was filled with a known quantity of water (6 liters) and two supporting rods were placed across it to support the Mett tubes. A thermometer was immersed in the water at the same level as the tubes. The water was then heated and the tubes placed into it in the following order, viz., one at 40° C. and others at 50°, 60°, 80°, 90°, and 100° C. respectively. At 70° C. two tubes were immersed, one of which was withdrawn when the temperature reached 75° C. and which I have labelled 70° X. The tubes placed in the bath at the different temperatures from 40° to 90° C. were kept in the water until the temperature of the bath reached 100° C. at which temperature, including the tube immersed at 100° C., they were left for five minutes.

In the preparation of these tubes, care was taken to avoid the inclosure of any air bubbles as their presence constitutes a source of great inaccuracy. Care was also taken to insure equal increments in the rise of the temperature of the water bath through equal periods of time. To have an equable temperature throughout the bath, the water was gently stirred.

The first change in the appearance of the egg-white was invariably noticed at 60.5° C. when it became faintly translucent and bluish. As the temperature continued to rise to the coagulating point of the albumin, the egg-white became whiter. The appearance of the coagulated egg white differed very markedly according as to whether it was immersed in the water at a low temperature and then gradually heated to the point of coagulation and beyond, or, whether it was immersed at a high temperature at the start. The former had a distinctly bluish tint varying according to the degree of heat, whereas the latter was creamy-white in color. It is comparatively easy, by observing the tinge of the coagulated material, to determine approximately at which temperature the tubes were placed in the bath.

From the tubes thus prepared, pieces about 4 cm. long were cut off and subjected to peptic digestion. For purposes of comparison, the pieces were suspended vertically in the following way: the 40° and the 100° were put in one bottle, the 50° and 90° in another, the 60° and 80° in a third and the 70° and 70° X in a fourth bottle. To each bottle, 15 cc. of the pepsin solution were added, as well as ten drops of chloroform to act as an antiseptic. The pepsin used was that prepared in scales by Parke, Davis and Company of which a 1 per cent solution was made in 0.4 per cent hydrochloric acid. Care was taken also that all the tubes were suspended in the digestive fluid at an equal level. Subsequently, experiments were made in which all the tubes were suspended in one bottle, each tube being placed in a vertical groove around a cork which was hung by a hook from the stopper. But no difference in the results was obtained.

The results showed a difference both as regards the zone of hydrochloric acid action and the zone of total digestion of the albumin; thus, the albumin gradually heated up from a low temperature was more acted upon, in both respects, than that which was immersed at a higher temperature, *i. e.*, the 40° albumin showed a greater zone of hydrochloric acid action and a greater zone of absolute clearness, equivalent to total digestion, than did the 100° albumin. The 50° was more acted upon, in the same way, than the 90° and the 60° more than the 80°.

Furthermore, the 40° albumin was acted upon to a larger extent than the 50° albumin; the 50° more than the 60° and so on up to the 100°.

The albumin which had been taken out at 75° C.—70° X—showed the greatest amount of change both in respect to the action of the hydrochloric acid and to the total digestion and was far in advance of all the others.

The following tables will bring out these points in figures. All of the results are expressed in millimeters.

TABLE I.

	40°		50°		60°		70°		70° X		80°		90°		100°	
HOURS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS
3..	5.3	1.0	5.0	0.8	4.7	0.7	4.4	0.6	6.7	2.9	4.1	0.5	3.7	0.3	3.5	0.2
6..	8.8	2.3	8.5	2.1	8.3	2.0	8.1	1.8	11.2	7.5	7.9	1.5	7.6	1.3	7.5	1.1
9..	12.0	4.0	11.6	3.7	11.3	3.4	11.1	3.1	15.6	12.2	10.8	3.0	10.6	2.7	10.3	2.5
12..	14.8	5.1	14.6	4.9	14.4	4.6	14.1	4.6	17.8	15.3	13.9	4.3	13.6	4.1	13.5	3.7
15..	15.7	6.8	15.5	6.7	15.2	6.5	15.0	6.1	18.0	17.1	14.7	5.8	14.4	5.5	14.0	5.3

TABLE II.

	40°		50°		60°		70°		70° X		80°		90°		100°	
HOURS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS
3..	6.0	1.1	5.4	1.0	5.0	0.8	4.6	0.7	8.0	3.0	4.0	0.6	3.6	0.5	3.0	0.4
6..	9.2	2.4	8.8	2.2	8.3	2.0	8.0	1.8	12.0	8.0	7.0	1.6	6.4	1.4	5.4	1.2
9..	12.6	3.5	12.0	3.3	11.5	3.1	11.0	3.0	15.6	13.0	10.0	2.8	9.2	2.4	8.0	2.0
12..	14.8	5.1	14.2	4.9	13.8	4.7	13.0	4.5	18.3	16.1	12.4	4.2	12.0	3.8	11.0	3.4
15..	16.4	6.7	16.0	6.5	15.2	6.3	14.4	6.0	20.0	19.2	14.0	5.7	13.4	5.2	12.0	4.8

TABLE III.

	40°		50°		60°		70°		70° X		80°		90°		100°	
HOURS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS
3..	6.0	1.2	5.4	1.0	4.8	0.8	4.2	0.7	8.2	3.3	3.8	0.5	3.4	0.3	3.0	0.1
6..	9.3	2.5	8.8	2.3	8.6	2.6	8.0	1.9	12.3	7.3	7.8	1.8	7.2	1.5	6.6	1.3
9..	12.4	3.8	12.1	3.6	11.6	3.3	11.2	3.0	15.4	13.5	10.8	2.8	10.2	2.5	9.6	2.0
12..	15.0	5.2	14.6	5.0	14.2	4.8	14.0	4.6	18.4	17.0	13.6	4.4	12.9	4.0	12.3	3.6
15..	17.0	7.2	16.6	6.8	16.1	6.0	15.8	5.6	21.1	19.7	15.5	5.4	14.8	5.0	14.0	4.8

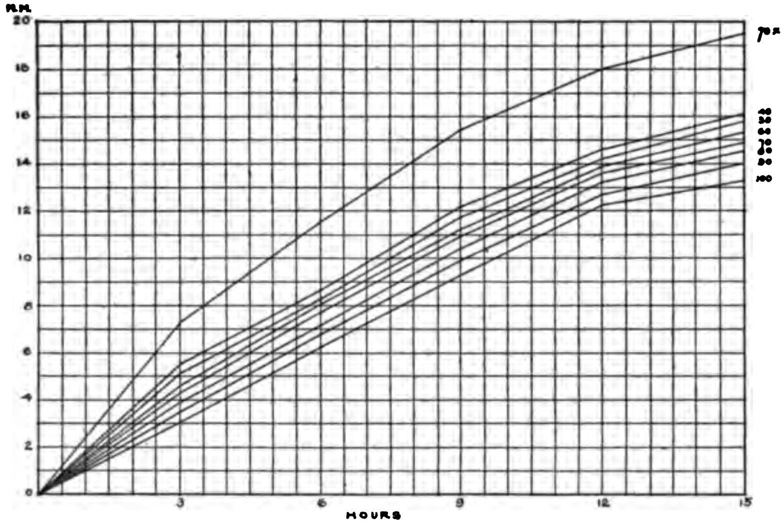
TABLE IV.

	40°		50°		60°		70°		70° X		80°		90°		100°	
HOURS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS
3..	5.8	1.0	5.4	0.8	5.2	0.6	4.6	0.4	7.5	3.0	4.3	0.3	4.0	0.2	3.6	0.1
6..	9.3	2.5	9.0	2.3	8.6	2.0	8.0	1.8	12.0	8.3	7.8	1.6	7.4	1.4	7.0	1.2
9..	12.6	4.0	12.4	3.6	11.8	3.4	11.2	3.2	16.5	13.6	11.0	2.9	10.7	2.6	10.2	2.4
12..	15.1	5.2	14.8	5.0	14.5	4.8	14.2	4.6	19.7	17.2	13.8	4.4	13.0	4.0	12.6	3.6
15..	16.4	7.0	16.2	6.6	15.8	6.4	15.5	6.0	20.5	19.1	15.0	5.8	14.5	5.4	13.9	5.0

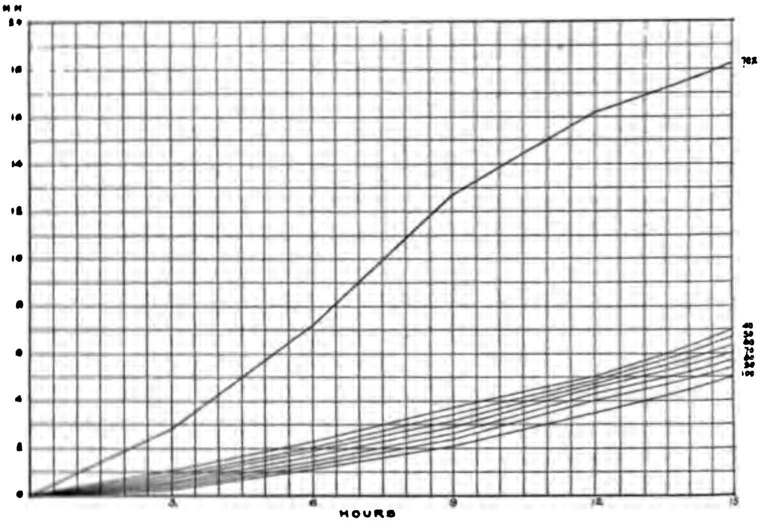
TABLE V.

HOURS	40°		50°		60°		70°		70° X		80°		60°		100°	
	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS	HCl	CLEARNESS
3..	4.5	1.0	4.1	0.8	3.9	0.6	3.5	0.5	6.0	2.0	3.3	0.3	3.0	0.2	2.6	0.1
6..	6.8	1.6	6.6	1.4	6.5	1.3	6.1	1.1	10.0	5.2	5.7	1.0	5.4	0.8	5.1	0.7
9..	11.0	3.2	10.6	2.8	10.1	2.5	9.8	2.2	13.9	11.0	9.3	2.0	9.0	1.8	8.4	1.6
12..	13.3	4.7	13.0	4.6	12.5	4.4	12.5	4.2	16.0	15.4	12.1	4.0	11.8	3.7	11.4	3.4
15..	15.0	7.2	14.6	7.0	14.0	6.4	13.6	6.0	18.0	16.6	13.4	5.6	13.0	5.3	12.6	5.0

On the basis of the tables here given I have prepared two mean curves, one representing the zone of action of the hydrochloric acid, the other the zone of total digestion.



CURVE 1. Showing zone of hydrochloric acid action.



Curve 2. Showing zone of total digestion.

From these curves it will be noticed that: 1. The progress of the hydrochloric acid action and the total digestion is most rapid in the albumin not heated beyond 75° C.

2. If the tubes were heated up to 100° C., then those in which the heating started at 40° show greater hydrochloric acid action and digestibility than those started at 50° and so on consecutively while the 100° shows the least.

3. The rate of digestion seems to progress more evenly with the hydrochloric acid action in the albumin heated up to 75° C. than it does in the others. Whereas the 70° X shows (according to the last reading given) 19.7 mm. hydrochloric acid zone and 18.3 mm. absolute digestion, the 40° shows 16.1 mm. hydrochloric acid zone and only 7.0 mm. absolute digestion.

4. The progress of the hydrochloric acid action and the digestion while proportionately greater the longer the period of digestion, diminishes relatively as digestion continues.

5. The ratio between the digestibility of the 40° albumin and the 70° X, from these curves, would be as 1: 2.6; while the ratio of the hydrochloric acid action would be 1: 1.2.

Corin and Ansiaux[1] and Hewlett[2] have shown that if white of egg is kept long enough at a temperature below that at which heat-coagulation occurs, the formation of opalescence and of flocculi will take place. And Halliburton[3] showed that this is due to the presence of a globulin in the egg-white which has a coagulating temperature below that of the egg albumin proper. It is stated that this coagulating temperature varies from 45° C. to 50° C. But as noted above, the first change observed in these experiments, owing to the short subjection of the white of egg to the heat, invariably took place at 60.5° C.

It has been shown microscopically by Bütschli and Hardy[4] and by Mann[5] that when a colloid separates out, an insoluble network or meshwork is formed in the interstices of which the solvent of the colloid is contained. The formation of globulin granules, we assume, acting as fixed points throughout the protein material causes the particles of coagulated albumin to be more finely and evenly distributed and makes it impossible for the albumin, on coagulation, to form coarse trabeculae. Hence, the egg-white gradually heated from a low temperature to its coagulating point shows a greater digestibility than that heated at once at the higher temperature. The difference in the color of the coagulated albumin depends upon the fineness and the homogeneity of distribution of the particles. The finer and more homogeneous the albumin is, the more translucent and bluish will it be. The coarser and less homogeneous it is the whiter and more opaque will it be.

The reason that the albumin labelled 70° X shows such marked increase in both hydrochloric acid and peptic action is due to the fact that the length of time of immersion in the water bath was insufficient to cause its complete coagulation. For ordinary purposes, however, such as for boiling eggs, this consistency of the albumin is quite sufficient and from a dietary point of view is very desirable and advantageous. That there is a difference in the homogeneity between the albumin heated only to 75° C. and the

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[1] *Bull. de l'academie roy. de Belgique*, xxi, p. 3, 1891.

[2] *Journal of Physiology*, xiii, p. 494, 1893.

[3] Schafer's *Text-Book of Physiology*.

[4] Mann's *Physiological Histology*, p. 122.

[5] *Ibid.*

40°, 50°, 60°, etc., is shown by the following experiment. Egg-white has a reducing power. For example, white of egg mixed with indophenol or with sodium sulphindigotate will cause a reduction of the dye on standing for several hours whether it be exposed to light or kept in the dark. Coagulation of the albumin causes a marked enhancement of this power of reduction. Mett tubes, made as described above except that in one series the egg-white was mixed with litmus, in another series with indophenol and in a third series with indigo-carmine, showed a rapid reduction of the dye while the tubes were still in the water bath. The degree of reduction varied. On the whole, it was greater in the tubes that were subjected for a longer time to the heat. On exposure to the air, the color of the dye would gradually reappear. The 70° X, however, showed the least reduction and, as compared with the other tubes, was distinctly more permeable to light. If the tubes were sealed in the flame before immersion in the water bath, no difference was seen. It is not, therefore, a question of the expulsion of the oxygen gas but of a reduction of the dye by probably traces of sugar in the egg albumin.

It is quite evident, from the results discussed in this paper, that the Mett tube method of standardizing ferments is an unreliable one unless special precautions be taken. In addition to the various objections that have been raised against it, such as the inclosure of minute air bubbles within the white of egg, and the settling of the egg-white within the tube before coagulation causing uneven layers, it must be stated that inasmuch as the digestibility of the perfectly fresh egg albumin will differ according to the temperature at which it is coagulated, the temperature at which coagulation takes place must be known before the strength of a ferment can be expressed in millimeters of digestion.

Further work will be undertaken to determine whether albumin placed directly into boiling water for different lengths of time, all evaporation being guarded against, will not also show differences in digestibility.

In conclusion I wish to thank Dr. Gustav Mann for his many helpful suggestions and criticisms throughout this work.

THE AMINO-ACIDS IN THE MATURE HUMAN PLACENTA.

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Interest in the amino-acids of the placenta has been stimulated by recent agitation concerning the manner in which the organ performs its physiological function, as well as by the part attributed to it, by some investigators, in the production of certain pathological conditions, more particularly in eclampsia. We have not hoped to solve either of these mooted questions, but we have felt that analysis of the organ by the newer methods of protein hydrolysis would be valuable in contributing to our knowledge of its fundamental composition, and perhaps in affording a logical basis for subsequent study of its physiology and pathology.

The placenta possesses an unique function and unusual importance; upon it depends the successful issue of every human pregnancy. Interpolated between the mother and the foetus, it is so constructed as to permit free passage of the blood of each without any possibility of the two commingling. Such an arrangement is not needed in species in which the egg, supplied with ample material for nourishment, leaves the mother before embryonic development begins, and only becomes necessary when the growth of the offspring occurs within the mother. Through the mediation of this organ, food reaches the foetus, and the waste products of its metabolism are returned to the mother.

A review of the architecture of the placenta will make this mechanism clearer. The placenta is a flat, more or less round structure, some 20 cm. in diameter and 2 to 3 cm. thick. Its weight averages approximately 500 grams, but varies with the size of the child, to which under normal conditions it bears a ratio of 1 : 6. Its two surfaces are designated as maternal and foetal, and are easily distinguishable, in that the umbilical cord is attached to the latter. Two greyish white, closely adherent membranes extend from the periphery of the placenta, and, unless artificially separated, give the impression of a single structure. The inner one, the amnion, encloses the amniotic fluid which surrounds the foetus during pregnancy; while the outer one, the chorion, is applied to the wall of the uterus. However, except for that portion of the latter which is specialized to form the placenta, these membranes have no interest in the present connection.

Microscopic study has demonstrated that very little maternal tissue is cast off with the placenta, namely a layer 1 to 2 mm. in thickness covering the maternal surface. The bulk of the organ is composed of delicate finger-like processes, the chorionic villi, which dip down into lakes of the mother's blood. The villi themselves are derived from foetal tissue, and enclose small blood vessels which only communicate with the large arteries and vein of the umbilical cord, and therefore, contain foetal blood. Between the two circulations, maternal and foetal, there is always interposed the solid wall of the villus, consisting of two strata, one epithelium, the other connective tissue. The outer of these, the epithelial, is continually bathed in the mother's blood. The connective tissue layer lies beneath the epithelial, which it supports, and in addition supplies a scaffolding that preserves the proper relations of the blood vessels, and maintains the form of the villus.

No channels run through the wall of the villus which might permit a direct means of communication between the blood of the mother and that of the foetus. All substances in transit from one circulation to the other must pass through both the epithelium and the connective tissue which compose the walls of the villi. Consequently, the part the villi play in the process has long been a prominent question, and one not yet answered satisfactorily. Two possibilities have been suggested; one that the wall is inactive, allowing passage in accord with the principles of osmosis and diffusion, the other that it is active, causing chemical changes in at least some of the material which passes through it. Definite proof has been adduced by Zuntz[1] and his pupils that gaseous bodies and an aqueous solution of sodium chloride pass from one circulation to the other in accord with the laws of physical chemistry. That a different process is concerned in the transmission of other substances would seem likely; and accordingly a number of investigators have brought forward experimental data which they believe indicate a digestive function in the placenta. A similar conclusion was reached by William Harvey[2] in 1651 upon purely theoretical ground.

Previous Investigations. The simplest analyses of the placenta have been concerned with the relative amount of water and of dried substance entering into its composition. The technique of these determinations has varied since some of the investigators have included the foetal blood in their material, and others have not. In either event, preparation of the material began with stripping off the umbilical cord, the amnion, and the membranous portion of the chorion as far as the periphery of the placenta. Following this, where it was not desired to remove the foetal blood, as in the analyses of Gaube[3] and others, the organ was cut in pieces and dried to a constant weight. Sfameni[4] and Grandis[5] expressed as much blood as possible from the organ by squeezing it between the hands. Higuchi,[6] very recently published two series of analyses, in one of which the material was unwashed, while in the other the foetal blood was removed by the perfusion of normal salt solution through the blood vessels. The fluid was injected by way of the umbilical vein, and escaped from the two umbilical arteries; about 12 liters of the solution were used for each placenta, but even after this, the washings were not perfectly clear.

The results, tabulated below, indicate that approximately 83 to 85 per cent of the unwashed placenta consists of water. An unusually large deposition of calcium salts increases the relative amount of solids. Thus, in calcareous placentae, Taltavall and Gies[1] found 80.25 per cent of water and 19.75 per cent of solids which were distributed as follows: 18.09 per cent organic and 1.66 per cent inorganic matter.

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[1] Zuntz: *Ergeb. d. Physiol.*, vii, p. 403, 1908.

[2] Harvey: *Exercitationes de generatione animalium*, London, 1651.

[3] Gaube: Essai de statique minerale du placenta et du foetus humain. Thèse de Paris, 1900.

[4] Sfameni: *Arch. ital. de biol.*, xxxiv, p. 216, 1900.

[5] Grandis: *Ibid.*, xxxiii, p. 429, 1900.

[6] Higuchi: *Biochem. Zeitschr.*, xv, p. 95; xvii, p. 21, 1909.

Water, Solids, and Ash in the Human Placenta.

AUTHOR	WATER	SOLIDS	ASH	REMARKS
	per cent	*per cent*	*per cent*	
Gaube............	85.33	14.16		Unwashed placenta: Male children
Gaube............	85.50	13.88		Unwashed placenta: Female children
Taltavall and Gies............	82.84	17.16	1.15	Unwashed placenta
Higuchi..........	84.90	15.09	0.88	Unwashed placenta
Sfameni..........	83.69	16.32	0.87	Blood squeezed out
Grandis..........	83.89	16.09	1.07	Blood squeezed out
Higuchi..........	88.65	11.35	0.71	Placenta perfused with salt solution
Grandis..........	88.80	11.20	0.795	Placenta perfused with salt solution

It is evident that chemical knowledge of the placental tissue itself cannot be secured unless the blood is removed and, on this account, the results obtained by Higuchi and by Grandis where the organ had been perfused with salt solution, offer the best idea of its composition. When the material for analysis has been so prepared, the relative amount of solids is perceptibly decreased and indicates that almost nine-tenths of the organ is water. Higuchi considers that his technique accounts in some measure for the unusually high water content, since some soluble material was carried out in the stream of salt solution passed through the organ about thirty times. On the other hand, it is possible that the tissue absorbed water. Although these conditions may have had some influence, it can be shown they are not entirely responsible for the high water-content of the organ.

Sfameni found that a given weight of foetal blood contains more solids than an equal weight of placenta. From this it follows that the retention of blood in the organ would increase the amount of solids there, and diminish relatively the amount of water. By way of contrast, Sfameni states that the percentage of water in the placenta, foetal blood and adult blood is respectively 83.68, 78.5, and 77.3 per cent.

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[1] Taltavall and Gies: *N. Y. Med. Journ.*, lxxxvi, p. 727, 1907.

Ash. Several attempts have been made to find a characteristic difference in the composition of the placenta according as the child was a male or female. The results are unconvincing. Gaube[1] states that whereas the minerals in the bodies of boys are in excess in those of girls, the opposite condition prevails in the placentae. In his analyses the placentae of males yielded an average of 2.164 grams, and those of females 2.255 grams of inorganic matter. Such figures are not sufficiently different to warrant the conclusion Gaube drew; moreover, from his own tables it appears there are many exceptions to the rule. The quantity of inorganic material which Gaube found in the placenta is about one-half that recorded by other investigators.

The results given in the foregoing table indicate that the ash is approximately one per cent of the total weight of the fresh placenta. The greater portion of this is soluble in water, according to Sfameni, who found the relation of the soluble to the insoluble ash as 6 to 1. Higuchi recently finds this relation, 4 to 3.

Obviously, the dried material obtained by driving off all the water would be relatively richer in minerals than the fresh placenta. Taltavall and Gies obtained 6.89 per cent of inorganic matter in dried normal placentae and 8.46 per cent in dried calcareous placentae.

The qualitative study of the placental ash has not been undertaken with sufficient thoroughness to yield reliable data. Gaube's analyses, which were the first reported, aimed at greater completeness than has since been attempted, but his methods were not refined, and his conclusions have never been corroborated.

Grandis[2] emphasizes the large amount of phosphates in the placenta, which he estimates as 33.46 per cent of the inorganic matter present; the phosphoric acid is in combination with calcium, magnesium and iron. His analyses of the ash also yield 11.4 per cent of chlorine; 24.9 per cent of sodium; and 6.57 per cent of potassium. An excess of sodium over potassium is unusual in human tissue; the only analogy, according to Grandis, occurs in the blood serum.

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[1] Gaube: *loc. cit.*

[2] Grandis: *loc. cit.*

Very recently, Higuchi[1] has estimated the total phosphorous and total sulphur in the placenta. These analyses indicate that the fresh, unwashed placenta contains 0.14 per cent of phosphorus and 0.12 per cent of sulphur; whereas, the corresponding figures with the foetal blood removed are 0.08 per cent and 0.06 per cent.

The Organic Constituents of the placenta have received more attention than its mineral matter but, notwithstanding, we have not, as yet, a clear idea of its organic composition. Work in this direction has been stimulated by interest in the toxaemias of pregnancy, for which a placental origin has been suggested. Those who favor this hypothesis believe that the toxins are of an organic nature.

Carbohydrates. The first organic compound to be demonstrated in the placenta was glycogen; Claude Bernard[2] identified it there in 1859, and subsequent investigators have confirmed his observation. With appropriate technique, it is now possible to localize the deposits of glycogen. Thus, applying microchemical methods, Driessen[3] has shown that in the mature human placenta glycogen is practically confined to the decidua. It is stored most abundantly in the neighborhood of the maternal blood vessels, and in the zone where the foetal and maternal elements commingle, that is, toward the surface of the decidua: very small amounts may be demonstrated at the periphery of the villi. Driessen has also noted that the amount of glycogen in the human placenta decreases as pregnancy advances. Analagous changes have been observed in the rabbit placenta by Lockhead and Cramer.[4]

Accurate, quantitative estimations of the glycogen cannot be secured by microchemical methods; to determine this, the glycogen must be extracted. That procedure was undertaken by Moscati,[5] who found that the placenta at full term contains from 2.5 to 3 grams of glycogen or from 0.49 to 0.58 per cent of its gross weight. Correct values are only obtained when the placenta is subjected to analysis immediately after its expulsion from the uterus since the glycogen-content rapidly diminishes from this moment. About one-half disappears within twenty minutes, and at the end of twenty-four hours no trace of it remains. Possibly, that explains the low figure obtained by Higuchi who found the glycogen in the placenta equivalent to 0.032 per cent of the weight of the organ.

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[1] Higuchi: *loc. cit.*

[2] Bernard: *Compt. rend. de l'Acad. d. Sci.*, xlviii, p. 77, 1859.

[3] Driessen: *Centralbl. f. Gynaekol.*, xxxii, p. 110, 1908.

[4] Lockhead and Cramer: *Proc. of Royal Soc., London*, lxxx.

[5] Moscati: *Zeitschr. f. physiol. Chem.*, liii, p. 386, 1907.

The disappearance of glycogen from the placenta may be due to one of two causes, namely contamination of the organ with bacteria; or the presence of an enzyme normally located there. The latter view is favored by Moscati, Opocher[1] and by Bergell and Liepman.[2] Moscati has found a ferment in the placenta which hydrolyzes glycogen and which bears a stronger resemblance to the enzyme found in muscle than to that in the liver. Opocher believes that the action of the ferment is reversible since he observed an increase in the amount of glycogen when the placenta was allowed to soak in a solution of grape sugar. On the other hand, Merletti[3] has passed a solution of glucose through the vessels of the placenta and noted that the returning fluid was poorer in sugar than the original solution. He regards a simple diffusion as impossible here since sodium phosphate perfused under similar conditions is returned quantitatively. Santi and Acconci[4] repeated the experiments of Merletti but were unable to verify his results.

Fats. Upon histological evidence the presence of fat in the placenta has long been admitted and Hofbauer[5] has recently studied its distribution very carefully by means of alcanna, osmic acid and soudan III. Hofbauer states that he was able to follow the stained globules passing through the walls of the villi toward the foetal circulation. The quantitative analyses of Higuchi, the only ones thus far published, indicate 0.846 per cent of fat in the unwashed and 0.535 per cent of fat in the washed placenta.

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[1] Opocher: *Annali di ostetr. e ginec.*, ii, p. 737.

[2] Bergell and Liepmann: *Münch. med. Woch.*, lii, p. 2211, 1905.

[3] Merletti: *Atti. soc. iz. ost. e ginec.*, ix, p. 122, 1903.

[4] Santi and Acconci: *Ginecologia*, p. 311, 1904.

[5] Hofbauer J.: *Gründzüge einer Biologie d. mensch. Placenta*, Vienna and Leipsig, 1905.

Higuchi also found 0.899 and 0.504 per cent of lecithin in the unwashed and washed tissue respectively.

Nitrogenous Compounds. Higuchi determined the total nitrogen (Kjeldahl) in the placenta as 2.226 per cent of the gross weight of the organ with foetal blood included. He found 1.331 per cent of nitrogen when the blood had been washed out. He tacitly assumes that all the nitrogen is present in the form of protein, for he multiplies the nitrogen content by 6.25 and states that the albumen of the unwashed and the washed placenta is 14.16 per cent and 8.32 per cent, respectively. Clearly, the calculation is inexact since a number of other nitrogenous bodies are present.

Nucleoprotein is the only albuminous body the isolation of which has been attempted from the placenta. Cocchi[1] assumed that the lesions in eclampsia are associated with coagulation of the mother's blood, and that this was due to the entrance of placental nucleoprotein into her circulation. Accordingly, in 1901, he sought to isolate this compound from eclamptic placentae, employing both the method of Wooldridge and that of Halliburton for this purpose. He considered that the substances obtained by these methods were identical; though the first gave a larger yield and was, therefore, permanently adopted. A small dose of the material thus obtained was fatal to rabbits; whereas, normal placentae treated in the same fashion furnished a material of very uncertain action and proved harmless in three of four experiments. Although the eclamptic material gave some of the color reactions for protein, the evidence adduced is insufficient to establish its identity. Thus, its composition was studied only with regard to phosphorus; two analyses yielded 1.839 per cent and 0.734 per cent respectively, facts which speak against the compound being a homogeneous one. Similarly, Bottazzi[2] sought a nucleoprotein in the placenta, but confined his study to the normal organ. Here again, it is uncertain whether the investigator was dealing with a compound or a mixture.

The most recent attempt to isolate nucleoprotein from the placenta was made by Kikoji,[3] who employed the method of Neuman.

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[1] Cocchi: *Lo sperimentale, Arch. d. biol. normale e patologica,* Firenze lv, p. 503, 1901.

[2] Bottazzi: *Boll. della R. acad. med. di Genova,* xviii, p. 245, 1903.

[3] Kikoji: *Ibid.,* liii, p. 411, 1907.

Kikoji obtained a substance which corresponded in its properties with the nucleoprotein of the thymus gland, and on decomposition yielded humin substances, levulinic acid, cytosin, thymin, guanin, xanthin, and hypoxanthin. Its elementary composition, however, was not constant.

Kikoji[1] in collaboration with Higuchi first isolated from the placenta the same purin bases that he later found in the nucleoprotein. Rielander[2] has also isolated uracil from the placenta and considers it a decomposition product of the nucleoprotein.

In 1901, Mathes[3] demonstrated albumoses in nine out of ten normal placentae by means of Devoto's method. His finding has been confirmed by Hofbauer[4] and by Basso.[5] Hofbauer believes that the albumoses result from the action of a proteolytic ferment which functions during life and is concerned with the passage of protein from mother to child. Having failed to find amino-acids in the fresh placenta, he believes digestion goes no further than the albumose stage.

The view that the splitting of protein is a normal function of the placenta has been endorsed by Ascoli[6] on very different grounds. This investigator employed the precipitin reaction, and found, after injecting egg albumen into a pregnant rabbit, that the activity of the maternal and the foetal blood in causing precipitation of a specific serum is not identical. He concluded, therefore, that the albumen injected into the mother was modified as it passed to the child. Savaré[7] agrees that a proteolytic ferment exists in the placenta, though its presence does not prove that the organ is other than a permeable membrane, for the enzyme may only have to do with the life processes of the placental cells.

Following Salkowski's[8] demonstration that various organs of the body will digest themselves when kept at 40° C. in sterile vessels, it was shown by Mathes that the placenta contains an autolytic enzyme. Subsequently a number of investigations have been made to determine the nature of the enzyme as well as of the end products resulting from its action. Thus, Ascoli,[1] Bergell in collaboration with Liepman[2] and Falk[3] have noted that the enzyme is "heterolytic" as well as "autolytic." It will split protein and peptones, though its action on gelatin and fibrin is very weak. Its analogy to trypsin is strong according to some investigators; while others consider that the enzyme is erepsin.

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[1] Kikoji and Higuchi: *Zeitschr. f. physiol. Chem.*, lii, p. 401, 1907.

[2] Rielander: *Centralbl. f. Gynaekol.*, xxxi, p. 1082, 1907.

[3] Mathes: *Ibid.*, xxv, p. 1385, 1901.

[4] *Loc cit.*

[5] Basso: *Arch. f. Gynaekol.*, lxxvi, p. 162, 1905.

[6] Ascoli: *Zeitschr. f. physiol. Chem.*, xxxvi, p. 498, 1902.

[7] Savaré: *Hofmeister's Beiträge*, ix, p. 141, 1907; *Ginecologia*, Firenze, iv, p. 34, 1907.

[8] Salkowski: *Zeitschr. f. klin. Med.*, *Festschrift f. Leyden*, xvii, Suppl., p. 77, 1890.

Dreyfus[4] has compared the "nitrogen distribution" in the fresh placenta and in the placenta after a week of autolysis. In the filtrate from the fresh material he finds that 4.8 per cent of total nitrogen is present as ammonia, 68.2 per cent as diamino compounds, and 46.2 per cent as monamino compounds; whereas, in the autolysed organ a corresponding analysis yields 9.2 per cent ammonia, 40 per cent diamino compounds and 61.3 per cent monamino compounds. Dreyfus infers there is a "desamidating ferment" in the placenta.

Leucine and tyrosine have been recognized as end products of placental autolysis. Mathes[5] discovered them in a placenta allowed to undergo autodigestion for a period of five and a half months. In repeating this experiment, Basso[6] recognized these monoamino acids through the appearance of the crystals, and substantiated the presence of tyrosine by obtaining a positive Millon reaction. This investigator noted more leucine than tyrosine, though he does not mention having separated the two substances and gives no analyses to establish their identity.

Thus far, no amino-acid has been isolated from the placenta. Rielander sought two of the hexone bases, lysine and histidine but was unable to detect them. The significance of the amino-acids has been made clearer by the demonstration that they are the fundamental compounds in the protein molecule. Moreover, improved methods of hydrolysis have placed in our hands the means of determining what amino-acids are present in a given material as well as of estimating their quantitative relation. Appreciable differences have already been demonstrated in the amino-acid constitution of various proteins, and it is not improbable that they are intimately associated with differences in physiological activity.

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[1] Ascoli: *loc. cit.*

[2] Bergell and Liepman: *loc. cit.*

[3] Bergell and Falk: *Münch. med. Woch.*, lv, p. 2217, 1908.

[4] Dreyfus: *Biochem. Zeitscher.*, vii, p. 493, 1908.

[5] Mathes: *loc. cit.*

[6] Basso: *loc. cit.*

EXPERIMENTAL DATA.

Our material has been obtained from the Obstetrical Department of the Johns Hopkins Hospital. All the placentae were normal and at full term. They were received at the laboratory within an hour after their delivery from the uterus, and were kept on ice until the following morning, when they were weighed, examined, and prepared for subsequent analysis.

The umbilical cord and the membranes were first removed. The membranous portion of the chorion, which forms the foetal surface of the placenta, and includes the large blood vessels, was carefully dissected off. Thus, there was left that portion of the chorion which contains the villi, together with a thin layer of decidua which covers the maternal surface of the placenta. Clearly this is the only part of the organ which has to do with the immediate transmission of substances to and from the foetus. Our interest has been to learn what amino-acids enter into the composition of the tissues concerned in the interchange. We have sought, therefore, to eliminate practically everything but the chorionic villi.

The blood was removed as completely as possible. Clots of maternal blood were easily wiped away. After some of the foetal blood was squeezed out by moderate pressure between the hands, the organ was cut into small pieces. These were rinsed in water, then thrown into a towel and wrung out. The process was twice repeated.

The weight of the material was again taken at this stage of the preparation and was found to represent 56 per cent of the original weight of the placenta. Since it is impossible to wring out the material with the same degree of thoroughness each time, there were variations in the different installments. The extremes met with were 37.5 per cent and 62.7 per cent. While not insisting on an absolute figure, it is of interest to note that approximately one-half of the weight of the placenta is accounted for by the foetal blood it contains.

The washed placental substance was next dried in an oven at 100° to 105° C. and after cooling to room temperature, was weighed. The various installments were mixed and finely powdered. This product furnished the material used for our analyses.

There were collected a total of 83 placentae; the sum of their original weights was 47220 grams. From these we secured 3320 grams of dry material which represents 7.03 per cent of the weight of the fresh organs. This ratio remained true, within comparatively narrow limits for all the installments.

Tabulation of Material Collected.

INSTALLMENT	NUMBER OF PLACENTAE	ORIGINAL WEIGHT	YIELD OF DRY SUBSTANCE	PERCENTAGE OF DRY SUBSTANCE
		grams	*grams*	
I	4	2350	161	6.8
II	2	930	55	5.9
III	4	3460	193	5.6
IV	6	3150	190	6.0
V	2	1100	85	7.7
VI	4	2080	173	8.3
VII	4	2280	197	8.6
VIII	8	4940	320	6.4
IX	4	2560	198	7.7
X	45	24270	1748	7.2
Total	83	47220	3320	7.03

Before proceeding with the analyses, we extracted the material with ether in order to remove fat and other ether-soluble matter. The material was then spread upon large sheets of filter paper and subsequently dried at 105° C.

Determination of Moisture. 1.5084 gram substance lost 0.0183 gram when dried to constant weight at 110° C. Therefore, 1.21 per cent of the material used in subsequent hydrolyses was moisture.

Determination of Ash. 1.5084 gram substance yielded 0.0992 gram ash. Ash = 6.58 per cent.

Determination of Nitrogen. 0.5840 gram substance gave $NH_3 = 57.4$ cc. $\frac{N}{10}$ H_2SO_4; N = 13. 76 per cent. 0.4842 gram substance gave $NH_3 = 47.6$ cc. $\frac{N}{10}$ H_2SO_4; N = 13.77 per cent. Calculated on ash- and moisture-free substance, N = 14.9 per cent.

Hydrochloric Acid Hydrolysis.

We have followed the method described by Emil Fischer.[1]

Five hundred grams of the powdered, ether-extracted material were hydrolyzed with boiling hydrochloric acid for twelve hours. This material s equivalent to 461 grams of the substance, free of ash and moisture.

The unhydrolyzed residue weighed 42 grams. Therefore, the weight of the hydrolyzed material was 419 grams.

After removal of the unhydrolyzed material, the filtrate was evaporated *n vacuo*. It was then esterified with three liters of absolute alcohol and saturated with gaseous hydrochloric acid. This liquid was inoculated with a few crystals of glycocoll-ester-hydrochloride and placed in an ice box for forty-eight hours. Subsequent examination failed to reveal any crystalization. Accordingly, the solution was again evaporated *in vacuo*, re-esterfied, and allowed to stand another forty-eight hours. This second attempt to isolate glycocoll-ester-hydrochloride was also unsuccessful.

The solution which contained the ester-hydrochlorides was next evaporated *in vacuo* at 40° C. It was treated in the usual way to set the esters free and the esters were extracted with a large quantity of ether. Having stood over night in contact with sodium sulphate, the ethereal extract was evaporated to an appropriate volume and then subjected to fractional distillation. The following fractions were obtained:

FRACTION	PRESSURE	TEMPERATURE	WEIGHT OF ESTERS
	mm.		*grams*
I	11	Up to 100° C	108.3
II	0.35	Up to 100° C	38.0
III	0.15	100° to 190° C	86.2
Residue			33.4

Proceeding with the treatment of these fractions according to Fischer we have isolated the following amino-acids.

Glycocoll. Although unable to isolate this body after primary esterification of the hydrolysed material, we have found it in the first fraction of the distillation-products from which it was isolated as the ester-hydrochloride.

Amount found was 5.75 grams, equivalent to 3.1 grams of free glycocoll.

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[1] **Emil Fischer: *Amleitung z. Darstellung organ. Preparate,* Braunschweig, 1905; Hoppe-Seyler: *Handbuch d. physiol. u. pathol. chem. Analyse,* 8th ed., Berlin, 1909.**

Analysis (Volhard): 0.2575 gram substance required 18.25 cc. $\frac{N}{10}$ $AgNO_3$

	Calculated for $C_4H_9O_2N.HCl$:	Found:
Cl	25.41	25.13

d-Valine. Amount found was 30.60 grams.

Analysis: 0.2062 gram substance yielded 0.3872 gram CO_2 and 0.1763 gram H_2O.

	Calculated for $C_5H_{11}O_2N$:	Found:
C	51.24	51.21
H	9.47	9.56

Specific rotation ($[\alpha]_D^{20}$) in 20 per cent hydrochloric acid (10 per cent solution) = +25.6° (±0.4°). Theoretical value = +28.8°.

l-Leucine. Amount found was 20.36 grams.

Analysis: 0.1865 gram substance yielded 0.3746 gram CO_2 and 0.1695 gram H_2O.

	Calculated for $C_6H_{13}O_2N$:	Found:
C	54.92	54.78
H	9.99	10.16

Specific rotation ($[\alpha]_D^{20}$)in 20 per cent hydrochloric acid (10 per cent solution) = + 14.8° (±0.4°). Theoretical value = +15.9°.

Proline. Both racemic and active prolin were isolated. Racemic copper proline: amount found was 1.63 grams. Equivalent in free proline is 0.81 grams.

Water of crystallization of copper salt was determined.

Analysis: 0.5902 gram dried at 108° to a constant weight lost 0.0645 gram H_2O.

	Calculated for $C_{10}H_{16}O_4N_2Cu+2H_2O$:	Found:
H_2O	10.99	10.93

l-Proline: amount isolated was 10.64 grams.

Analysis: 0.1896 gram substance yielded 0.3475 gram CO_2 and 0.1316 gram H_2O.

	Calculated for $C_5H_9O_2N$:	Found:
C	52.14	50.0
H	7.88	7.76

The analysis indicates that the l-proline was impure. Several attempts to purify it were futile. Hence, the amount of pure l-proline was determined according to the optical rotation of the various fractions which had been obtained. Thus corrected, the amount of l-proline found was 7.17 grams.

The optical rotation of the analysed substance was determined in a 10 per cent aqueous solution ($([\alpha])^{20}_{D}$ = −57.1° (±0.2°). The theoretical value for pure l-prolin = −81.5°.

Phenylalanine. Amount isolated as the hydrochloride was 12.26 grams. Equivalent in free phenylalanine is 10.08 grams.

Analysis (Volhard): 0.3079 gram of the hydrochloride required 15.05 cc. $\frac{N}{10}$ $AgNO_3$.

	Calculated for $C_9H_{11}O_2N.HCl$:	Found:
Cl	17.59	17.33

Since the material was inactive to polarized light, it must have consisted of the racemic acid.

Aspartic Acid. Amount of the racemic acid found was 9.44 grams.

Analysis: 0.2066 gram substance yielded 0.2741 gram CO_2 and 0.0985 gram H_2O.

	Calculated for $C_4H_7O_4N$:	Found:
C	36.07	36.18
H	5.30	5.33

Some impurity prevented the isolation of the active acid.

d-Glutaminic Acid. Amount isolated as the hydrochloride was 15.91 grams. Equivalent in free glutaminic acid is 12.68 grams.

Analysis (Volhard): 0.1874 gram substance required 10.30 cc. $\frac{N}{10}$ $AgNO_3$

	Calculated for $C_5H_8O_4NHCl$:	Found:
Cl	19.21	19.48

The specific rotation was determined in dilute hydrochloric acid ($[\alpha]^{20}_{D}$) = + 27.5° (± 0.2°). Theoretical value = + 30.5°.

Alanine. It was impossible to isolate alanine: if present, it occurs in extremely small quantity.

l-Tyrosine. We have made a separate hydrolysis with sulphuric acid to determine the amount of tyrosine in the placenta.[1]

Two hundred and fifty grams of the powdered ether extracted material was taken. This is equivalent to 230.5 grams ash and moisture-free substance. Non-hydrolyzed material weighed 26.3 grams. Amount of tyrosine found was 3.86 grams.

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[1] Emil Fischer: *loc. cit.*, p. 88.

Analysis: 0.2092 gram substance yielded 0.4567 gram CO_2 and 0.1186 gram H_2O.

	Calculated for $C_9H_{11}O_3N$:	Found:
C	59.64	59.54
H	6.12	6.34

The specific rotation was determined in 4 per cent hydrochloric acid (5 per cent solution). ($[\alpha]_D^{20°}$) = −11.3° (± 0.2°). Theoretical value = −13.2°.

Determination of Hexone Bases.

The method used was that of Kossel and Kutscher.[1] Fifty grams of the powdered ether-extracted material, equivalent to 46.1 grams ash-and moisture-free substance, were taken.

Lysine. Amount isolated as the picrate was 4.10 grams, equivalent to 1.6 grams of free lysine.

Analysis: 0.2049 gram substance yielded 0.2888 gram CO_2 and 0.0882 gram H_2O.

	Calculated for $C_{12}H_{17}N_5O_9$:	Found:
C	38.4	38.44
H	4.53	4.81

Arginine. Amount isolated as the mononitrate was 3.25 grams. This was recrystallized from 85 per cent alcohol, but a combustion revealed that the material was still impure. Consequently, it was converted into the methylester hydrochloride.[2]

1.62 grams mononitrate yielded 1.49 grams methyl-ester hydrochloride. Total equivalent in free arginine is 2.00 grams. Melting point of the ester hydrochloride isolated was 193° (uncorr.).

Analysis (Volhard): 0.2027 gram required 15.35 cc. $\frac{N}{10}$ $AgNO_3$.

	Calculated for $C_7H_{16}N_4O_2.2HCl$:	Found:
Cl	27.16	26.85

Histidine. Amount of crude substance isolated as the dichloride was 1.66 grams. This material was recrystallized from hydrochloric acid; even then the analysis did not yield the theoretical values. By persistent recrystallization 0.166 grams of crystalline histidine dichloride was isolated from the crude material. Its melting point was 223-225° (uncorr.).

Equivalent of free histidine is 0.11 gram.

[1] *Hoppe-Seyler's Handbuch d. chem. Analyse,* p. 516, 1909.

[2] *Berichte,* xxxviii, p. 4173, 1905.

Analysis (Volhard): 0.1450 gram substance required 12.55 cc. $\frac{N}{10}$ $AgNO_3$.

	Calculated for $C_6H_9N_3O_2.2HCl$:	Found:
Cl....................................	31.1	30.7

Tryptophane. The presence of tryptophane was demonstrated as follows: one gram of the crude placental powder was allowed to digest with 10 cc. of a 2 per cent solution of trypsin. The filtered solution gave an intense violet color with bromine water. Control tests with the placental powder and the trypsin solution itself were made simultaneously.

Ammonia. The hydrolyzed substance contained 1.28 per cent ammonia (Hart's method).[1]

The placental material used for the hydrolyses contained 92.2 per cent of organic matter and approximately one-third of this is accounted for by the products we have isolated. In view of the fact that Fischer's method of hydrolysis fails to give a theoretical yield even when isolation of known mixtures of amino-acids is attempted, one could not expect to recover the amino-acids quantitatively from the placenta. To determine the accuracy of the ester method, Osborne and Jones[2] who have made such mixtures, have shown that the yield varies between 41 per cent and 82 per cent of the theoretical quantity. Moreover, they have worked under conditions which practically assure complete esterification.

We have not attempted the isolation of cystine, serine, oxyproline and tryptophane; though the presence of the last was demonstrated after tryptic digestion by the bromine reaction. We have made a careful search for alanine but were unable to isolate it. While not inclined to insist absolutely upon its absence, we are confident that, if present at all, extremely small amounts occur in the placenta.

An excess of valine over leucine can hardly be attributed to a peculiarity in the composition of the placenta. Levene and Van Slyke[3] have found that the quantity of valine in several proteins is greater than was formerly thought.

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[1] Hart: *Zeitschr. f. physiol. Chem.*, xxxiii, p. 347, 1901.

[2] Osborne and Jones: *Amer. Journ. of Physiol.*, xxvi, p. 305, 1910.

[3] Levene and Van Slyke: this *Journal*, vi, p. 419, 1909.

Contrary to the opinion of Rielander[1] lysine and histidine does occur in the placenta, though only a very small quantity of the latter was isolated. Arginine is the most abundant of the hexone bases.

Our hydrolytic products account for about two-fifths of the total nitrogen of the placenta ranked according to the nitrogen they account for, the monamino-acids take the following order: valine, leucine, glutaminic acid, proline, aspartic acid, phenylalanine, tyrosine and glycocoll.

SUMMARY.

I. Approximately one-half of the fresh placenta is blood.

II. The dried placental material is about 7 per cent of the gross weight of the organ.

III. The hydrolytic products represent at least 31 per cent of the organic matter.

IV. Placental tissue, free of ash and moisture contains 14.9 per cent of nitrogen.

V. The nitrogen in the isolated products represents 5.6 per cent of the weight of the material used for hydrolysis.

VI. The relative amounts of the amino-acids in the placenta are as follows:

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[1] Rielander: *loc. cit.*

	AMOUNT IN 461 GRAMS PLACENTAL MATERIAL, FREE OF ASH AND MOISTURE	PERCENTAGE OF PLACENTAL MATERIAL	NITROGEN AS PER CENT OF PLACENTAL MATERIAL
	grams		
Glycocoll	3.10	0.63	0.118
Alanine	?	?	?
Valine	30.60	6.64	0.794
Leucine	20.36	4.42	0.473
Proline	7.98	1.73	0.228
Phenylalanine	10.08	2.19	0.186
Glutaminic acid	12.68	2.75	0.262
Aspartic acid	9.44	2.05	0.216
Tyrosine	7.72	1.68	0.130
Lysine	16.00	3.46	0.664
Arginine	20.00	4.33	1.394
Histidine	1.10	0.24	0.064
Tryptophane		present	
Ammonia		1.28	1.054
		31.40	5.583
Nitrogen in non-hydrolyzable substance			0.146
			5.729

A BIOCHEMICAL STUDY OF PROTEUS VULGARIS HAUSER.[1]

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In this study we deal with the cultural properties, the products of growth, the pathogenicity, and the toxicity, of two strains of *Proteus vulgaris*, Hauser. This is an exceedingly interesting organism on account of its wide distribution, the ease with which it is modified, and because it seems to stand between the true saprophytic and the purely pathogenic bacteria. It has been found under a great variety of abnormal conditions in the human body, but most frequently in the digestive tract, where it produces the greatest harm. Here it has been present in large numbers, in a variety of food poisonings (1, 2),[2] in the summer diarrhœas of children (3, 4), the diarrhœas of calves (5), in severe cases of typhoid fever (6), and in other more obscure abnormal intestinal conditions.

Its association with other pathogenic organisms is of great importance and probably has not received the attention it deserves. Theobald Smith (7) found that when the hog cholera bacillus and Proteus were grown together, in what he called a mixed pure culture, the virulence of the former was lowered, while that of the latter was apparently raised. Kühnau (8) found virulent strains of Proteus in severe cases of diphtheria. Levy and Thomas (9) find that the virulence of the cholera spirillum was raised by injecting cultures of Proteus precipitated by alcohol. Its presence in severe cases of typhoid has already been mentioned (6) and in the same article Vincent notes that mixed cultures of Proteus and the typhoid bacillus are very virulent for rabbits.

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[1] Aided by a grant from the Rockefeller Institute for Medical Research.

[2] Figures in parenthesis refer to bibliography at the end of this paper.

DESCRIPTION OF CULTURES.

Both the cultures used in this study were obtained from Dr. Theobald Smith. Culture A had been in his possession for some time, while Culture B was isolated from some putrid material in January, 1910. Culturally these two strains do not agree in all points, but they will be described together and their differences noted.

Morphology. Both organisms are short rods which vary considerably in length. They are motile, do not produce spores, and are negative to Gram's stain. In Culture A the organisms occur in long chains while in Culture B they are isolated.

Cultural characteristics. On an agar slant there is a moderate, spreading, glistening growth covering the entire surface of the media. The condensation water is turbid. In bouillon there is an abundant growth with a small amount of flocculent white sediment. Culture A shows a tendency to form a surface membrane.

When it was received, Culture A liquefied gelatin slowly and when plated it was found that the majority of the colonies were non-liquefiers. The liquefying colonies were of the swarming type so well described by Hauser (10) and Smith (11). Subcultures were made from the latter and from time to time the culture was plated and subcultures always made from the liquefying colonies. In May, 1910, it was found that the culture did not digest casein, but its action on gelatin was not tested until the following July when it was found that there was no sign of liquefaction in a stab culture in three weeks' time. In August another culture of this strain was received from Dr. Smith and it was found that the liquefying properties were the same as when the culture was first studied by us.

Culture B liquefied gelatin rapidly throughout the period in which it was studied, although it was treated in exactly the same way as Culture A. In July, 1910, a stab culture was liquefied to a depth of 5 cm. in twelve days. Swarming colonies were observed in one instance only, in which case the culture had been passed rapidly through a series of milk tubes and then plated on glucose gelatin. However, the culture was not plated on 5 per cent gelatin and it is probable that on this medium swarming colonies would have been found.

Culture A was unfortunately not studied in milk at the time (April) it was received, but in July it caused no apparent change in twelve days. The reaction at the end of this period was about +1.5 per cent.[1]

Culture B in milk shows no apparent change in two days but on the third day there is a soft curd and signs of digestion. In five days the milk is about five-eighths digested and the reaction is about +2.5 per cent. In a milk fermentation tube the bulb is very slowly acted upon, being half digested in twelve days.

Dr. Theobald Smith has repeatedly pointed out the fundamental character of the fermentative power of Proteus and of other organisms. He says (12),

> In view of this confused state of affairs (the various forms of Proteus) I would suggest that in the study of doubtful forms we should apply the fermentation test and class those species which fail to act on lactose, but which ferment dextrose and saccharose . . . . under Proteus. I found the fermentation test such a valuable group reaction, that I can safely recommend it in the study of all saprophytic forms in supplying a broader view of the relationship of physiological species.

If one follows his method, taking into consideration the amount of acid formed in the bulb and branch, the information one gains is truly remarkable. In different organisms of the same species one may get variations in the reaction of the bulb due probably to different rates of growth. In the branch, however, the amount of acid produced is very typical of the species. It is to be hoped that before long we will have a classification of bacteria based on the growth in the fermentation tube, as this seems to be the surest and easiest means of differentiating a great many species.

As Dr. Smith has shown, Proteus forms from 20 to 30 per cent of gas in dextrose bouillon and the acid produced is between 2 and 3 per cent of a normal solution.

In lactose one sometimes gets a small bubble of gas but as this is so rare, it may be due to some other sugar in the media. The reaction of the media is near the neutral point.

The production of gas in saccharose is slow, often not beginning before the third or fourth day, but it amounts to 25 or 30 per cent in ten days. The amount of acid produced is smaller than in the case of dextrose, being from +1.5 to +2.0 per cent. In some cases the reaction in the bulbs is low but in the branch the reaction is very constant.

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[1] In this paper + represents acidity and − represents alkalinity, the reaction being determined by titrating 5 cc. of the diluted and boiled media with $\frac{N}{10}$ NaOH.

It will be seen that the only constant distinctive cultural characteristics shown by these two strains are the fermentative powers. Yet it was found that they formed practically the same chemical products and that their pathogenic powers were the same. Culture A, after being passed through a mouse, liquefied gelatin and resembled typical *Proteus vulgaris*.

An interesting point brought out was the cause of the coagulation of milk by these organisms. Smith (12) says that

> The contemporaneous power to liquefy gelatin and precipitate casein in milk indicates that the two processes are due to a ferment, and that the changes produced by *Proteus vulgaris* in milk are not the result of an acid fermentation of the milk sugar, as is the case with the colon group.

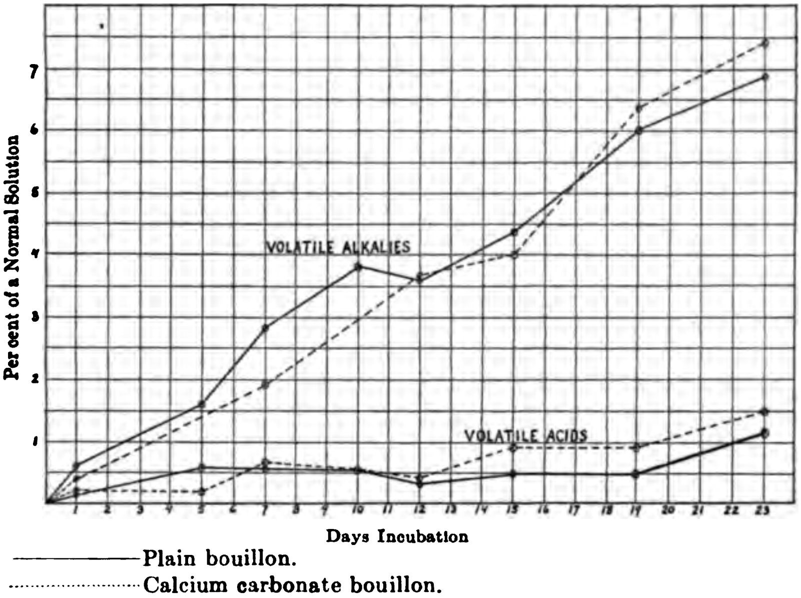
Roger (13), on the other hand, admitting that milk sugar is not fermented, notes that when the casein is precipitated the media is acid and thinks the acid is responsible for the curdling.

We noticed that there was a greater amount of acid produced by the peptonizing strain of proteus in milk and it was thought that this might be the cause of the coagulation. However, on the addition of 0.5 per cent of calcium carbonate to the media the coagulation was more prompt and the digestion more rapid than in the control tube, while the reaction in the calcium carbonate tube when coagulation took place was +1.5 as against a reaction of +2.8 in the control. When 1 per cent magnesium carbonate was added to the milk, the acid formed was all neutralized and here also the coagulation was more prompt and firmer than in the plain milk. Coagulation, then, must be due to an enzyme which acts best in a neutral or slightly acid medium.

PRODUCTS OF GROWTH.

A bouillon containing 0.4 per cent of Liebig's beef extract, 1 per cent of Witte's peptone and 0.5 per cent of sodium chloride was used in studying the products of growth of these organisms. No differences could be found between the two strains, so that the following notes apply to both cultures.

Lewandowsky (14), using plain bouillon, reports *Proteus vulgaris* as an organism that forms phenol. We were never able to detect this substance in the distillate from acidified cultures, although a number of cultures were examined, including a fifteen-day culture in 5 per cent peptone bouillon and a sixteen-day culture in bouillon containing 0.02 per cent of tyrosin (15). Skatol has also been reported as one of the products of growth of *Proteus vulgaris*, but using the delicate paradimethylamidobenzaldehyde (Ehrlich's aldehyde) test, we were never able to find it in any of our cultures, including those mentioned under phenol.



Mercaptan was absent, even when cystine had been added to the media. Alcohols, aldehydes and ketones could not be found.

Indol was present in moderate amounts but seldom in concentrations sufficient to give a precipitate with β-naphthaquinone-sodium-monosulphonate. Indol-acetic acid was present in cultures four days old, it being identified by its color reaction with concentrated hydrochloric acid and a few drops of 0.01 per cent sodium nitrite, its spectrum, and its color reaction with hydrochloric acid and ferric chloride (16). Aromatic oxyacids were present in moderate amounts. Hydrogen sulphide was present even in twenty-four-hour cultures.

Volatile alkali is produced in large amounts as is shown in the curve on the preceding page. This is probably for the most part ammonia, but primary amines are present as shown by the carbylamine test. Putrescine and cadaverine could not be demonstrated in cultures three weeks old.

In estimating the volatile alkali and acids a series of flasks containing 60 cc. of bouillon and with paraffined plugs, was inoculated and incubated at 37° C. From time to time a flask was opened and 25 cc. taken out for each determination. A mark placed on the outside of the flask at the surface of the media showed that the loss by evaporation of the media was so slight that it could not be detected. In order to liberate the alkali, 25 cc. of the culture was diluted and about 1 gram of magnesium carbonate added and the volatile alkali determined as in the Kjeldahl method. The volatile acids were liberated by the addition of about 1 cc. of phosphoric acid to another 25 cc. of the culture, and after distilling, the distillate was titrated with $\frac{N}{10}$ sodium hydrate.

The volatile acids are small in amount but their molecular weight is remarkably high as will be seen in Table II.

TABLE II.

AGE OF CULTURE	MOLECULAR WEIGHT OF VOLATILE ACIDS		
	Proteus A	Proteus B	Bacillus Coli
7 days	84	100.3	
14 days	97.5	102	78.5
25 days	100.3		

When the organisms were grown in flasks containing 1 per cent casein and 0.06 per cent sodium carbonate, the culture precipitated with Hedin's tannic acid mixture (17), and the soluble nitrogen determined in the filtrate, it was found that Proteus A had failed to act on the casein, while Proteus B destroyed 64.2 per cent of the protein in three weeks' time. The failure of Proteus A to act on the protein corresponds with its loss of power to digest gelatin. When to the casein solution was added 1 per cent of dextrose or lactose, the protein was not attacked by Proteus B. This is rather peculiar, as in a fifteen-day milk culture of this organism there was 18.1 per cent more soluble nitrogen than in the control milk. We failed to get any action on a 1 per cent solution of dried egg albumin but there was apparently little growth and it seems probable that with the addition of some salts a more suitable medium could be made in which the protein would be acted upon.

The study of the products of the growth of Proteus shows that, to some extent at least, it is a putrefactive organism. It destroys a native albumin (casein) and it produces ammonia, primary amines, hydrogen sulphide, fatty acids of a high molecular weight, aromatic oxyacids, indol and indolacetic acids, all of which are associated with putrefaction. Given the proper conditions in the digestive tract, it would probably prove harmful to the host through the formation of these substances (18).

PATHOGENIC PROPERTIES OF PROTEUS.

Injected under the skin of guinea-pigs, bouillon cultures of these organisms caused large ulcers which healed slowly. When injected into the peritoneal cavity, the weight of a guinea-pig fell from 575 to 301 grams in sixteen days, and the animal died apparently from starvation and a diffuse peritonitis.

In mice the lethal dose of a twenty-four-hour bouillon culture was about 1 cc., but by passing the cultures through seven mice, the virulence was raised so that the lethal dose was 0.1 cc. for Proteus A and 0.25 cc. for Proteus B.

Young guinea-pigs weighing 200 grams were fed, for a week, on a cake made of bread and 7 cc. of a twenty-four-hour bouillon culture of the organisms. They lost a few grams in weight but this was probably due to their somewhat limited diet rather than to the cultures.

Nursing kittens three days old were fed bouillon cultures as follows, the cultures being injected into their stomachs through a catheter:

August 1 each kitten received 0.5 cc. of a 24-hour culture.
August 3 each kitten received 1.0 cc. of a 24-hour culture.
August 4 each kitten received 1.0 cc. of a 24-hour culture.
August 5 each kitten received 1.0 cc. of a 24-hour culture.
August 6 each kitten received 5.0 cc. of a 24-hour culture.

Kitten I received cultures of Proteus A, the virulence of which was 0.1 cc. for mice. Kitten II received cultures of Proteus B, the virulence of which was 0.25 cc. for mice, and Kitten III, the control, received plain bouillon. The kittens showed no apparent effects from the cultures throughout the experiment nor during the following month.

Two monkeys (*Macacus rhesus*) on a diet of eggs and corn-meal were fed daily for a month, 50 cc. of twenty-four-hour cultures of Proteus A and B, the virulence of which for mice was 0.1 cc. and 0.25 cc. respectively. No ill effects were noticed in either monkey during or following the feeding, although the organisms fed were found in the feces of both monkeys.

A monkey (*Macacus rhesus*) on a diet of meat and eggs was fed 50 cc. daily of a twenty-four-hour bouillon culture of Proteus B. This caused a marked diarrhœal stool of a greenish color. The organisms were found in the feces in great numbers for the first two weeks but after that about two in ten colonies were Proteus. After a month and a half, the monkey became very weak and was chloroformed. The walls of the cæcum, ascending and transverse colon were thickened and congested. Small ulcers, about 4 mm. in diameter, were scattered over this area. The remaining parts of the digestive tract were apparently normal. Cultures from the spleen and blood were sterile. The blood showed a slight agglutinative reaction toward the organism fed, there being a microscopic agglutination in dilutions of 1:50. Spectroscopic examinations of the blood made from time to time showed it to be normal.

The results of these feeding experiments are rather indefinite and correspond to those recorded in the literature. Vincent (19) and Schumburg (20) state that cultures of Proteus isolated by them from the vomited material of patients suffering from meat-poisoning caused diarrhœa and death when fed to mice. Myerhof (21), on the other hand, says that his cultures of Proteus, when fed to mice, caused neither sickness nor immunity.

Metschnikoff (22), working with proteus, isolated from cases of summer diarrhœa in children, failed to get any effect when the cultures were fed to young macacus and cynocephalus monkeys. When fed to nursing rabbits and young chimpanzees, a characteristic diarrhœa resulted which caused the death of the former.

TOXIN.

Following the method used by Levy (23) and Vaughan (24) we precipitated cultures of proteus by adding alcohol to the strength of about 70 per cent, centrifugalizing and drying the precipitate in a vacuum over sulphuric acid. When dry the substance is of a brownish color and has a slightly putrid odor. The yield is about as follows:

500 cc. bouillon 8 day culture at 37° = 0.48 grams.
500 cc. gelatin 7 day culture at 37° = 1.41 grams.
Growth from agar slants in 3-quart bottles incubated 7 days at 37° = 0.5 grams.

The material used for most of the work was obtained from seven-day growths on agar slants, as it was found that this was the easiest and quickest way of working with the cultures.

The precipitate obtained is sparingly soluble in salt solution, 1 part dissolving in about 200 parts of physiological salt solution. It is soluble in 1 per cent sodium carbonate; precipitated by alcohol, $\frac{N}{20}$ hydrochloric acid, magnesium sulphate, ammonium sulphate; is not coagulated by heat, gives the xanthoproteic, Millon's and biuret reactions.

It is completely digested by trypsin but only partially by pepsin. The substance obtained from gelatin cultures gave 15.4 per cent nitrogen, while that from agar cultures gave 9.96 per cent nitrogen. Prepared in this way, the precipitate probably consists largely of nucleoprotein but other substances are undoubtedly present.

When dissolved in sterile salt solution and injected into the peritoneal cavity of guinea-pigs, this substance caused the death of the animal in from six to twenty hours, the lethal dose per 100 grams of body weight being as follows:

Precipitate from gelatin culture, 11.5 mg.
Precipitate from bouillon culture 9 mg.
Precipitate from agar culture, 8.2 mg.

After a fatal dose is injected, the symptoms are as follows: The pig quickly appears to be very sick, has a staring coat, and remains quiet in the corner of its cage. Soon it lies on its side, as though in an exhausted condition and its temperature falls often as low as 32° C. No evidence of pain or convulsions were observed when the injection amounted to 8 or 10 mgs. per 100 grams of body weight. When larger amounts were given, the animal cried out and there were spasmotic movements of the abdominal muscles, as though the animal would vomit, although this act never took place in a guinea-pig.

At autopsy it was found that the walls of the peritoneal cavity are congested and small punctiform hæmorrhagic spots are often present. In the cavity there is a decided increase in the amount of straw-colored fluid, often tinged with blood. The liver, spleen, stomach and intestines are covered by a fibrin exudate, rich in polymorphonuclear leucocytes. The liver is swollen and somewhat darkened. The spleen is swollen, darkened and often shows on the surface hæmorrhagic spots, the size of a pin-head. The adrenals show a surface congestion, while the kidneys are apparently normal. The vessels of the digestive tract are often enlarged and the stomach and cæcum contain varying amounts of gas depending upon the length of time the animal has lived after the inoculation. In the pleural cavity the lungs appear to be normal, while the vessels of the heart are engorged with blood and under the visceral pericardium small hæmorrhagic spots are often to be seen.

Microscopical examination of the tissues shows a slight granular degeneration of the liver, accompanied, at times, by a perilobular lymphocyte infiltration. In some animals there was a marked degeneration of the liver which was probably dropsical, as the cells showed large vacuoles. The kidney shows a slight granular degeneration. The spleen shows marked congestion with a distension of the sinuses, while the lungs and adrenals are apparently normal. In the heart the capillaries are greatly distended with blood and at times it seems as though they had burst, causing hæmorrhage between the muscle fibers.

What would be a fatal dose if injected into the peritoneal cavity, when given under the skin does not produce these results. There is a slight rise in temperature and the loss of ten grams or so in weight, this loss, however, being soon regained. An induration is felt in the subcutis and in about three days the hair comes out and an ulcer is formed. The ulcer is at first small but after several days the opening is about 1.5 cm. in diameter and extends down to the muscular layers, with a sharply circumscribed edge. There is a slight discharge, and a thick, yellowish pus can be seen in the depths of the ulcer. The healing process is very slow, extending over a period of about three weeks. The same type of lesion is produced by 1 cc. of a twenty-four-hour culture of Proteus and by the bodies of the bacteria killed by heat.

In white mice the lethal dose lies between 2.5 and 5 mgs, the mouse dying in from five to twelve hours. The macroscopical changes noted are a mottling of the liver, a congestion of the spleen, adrenals and at times, of the kidneys. The contents of the intestine are fluid, and gas is often present. The heart is very much engorged and its vessels are dilated.

In cats the picture is as follows:

August 4, 1910. 10:15 a.m. Cat weighing 1740 grams anæsthetized with chloroform and 30 mg. toxin No. 15 in 10 cc. sterile salt solution injected into a superficial vein of the leg.

10:50 a.m. Cat crying out and vomits a considerable amount of partly digested food.

12:30 p.m. Cat seems brighter but from time to time makes violent attempts to vomit and is unsteady on its feet.

5 p.m. Cat rouses up when disturbed but if left alone it sits in a corner with its eyes closed and seems to feel very badly.

August 5. 8 a.m. Cat found dead, stiff. Autopsy at once.

Peritoneal Cavity. Liver apparently swollen and mottled. Gall-bladder partly filled with bile. Spleen swollen and bluish in color.

Kidneys. The vessels on the surface of the organ and those going to it are distended with blood. On section there seems to be a congestion of the medullary rays.

Adrenals. Small and apparently normal.

Stomach. Very much distended with gas. Large scattering hæmorrhagic areas lay in the mucous membrane near the pylorus.

Intestines. Moderately distended with gas. No hæmorrhagic areas found.

Pleural Cavity. Lungs apparently normal. Heart congested and shows small hæmorrhagic areas under the visceral pericardium.

No bacteria could be found in smears from the blood or spleen.

Cat No. 2.

August 22, 1910. Cat weighing 3525 grams anæsthetized and 15 mg. toxin No. 17 digested twenty-four hours with trypsin injected into the femoral vein.

10:20 a.m. Cat feeling badly. Strings of saliva hanging from mouth. No vomiting.

12. Noon. Cat has had nothing to eat since yesterday morning. Vomits a greenish substance containing bubbles of gas. Looks very sick. During the afternoon the cat vomited twice and seemed very sick.

August 23. 8 a.m. Large amount of bloody diarrhœal feces in cage. Cat slept all morning and refused to eat.

August 24. No feces. Cat still sleeping most of the time and refuses to eat.

August 25. Cat feeling better but eats very little. Weight 3180 grams. From this time on the cat returned to normal.

When sublethal doses are injected into the peritoneal cavity of a guinea-pig it often is found at the end of twenty-four hours that part of the rectum is protruding from the anus. This might indicate that the substance causes an increased peristalsis and it was found that in one of these animals which was autopsied immediately after chloroforming that the peristaltic movements were very marked.

The properties of this toxin were studied to a certain extent, although we have not been able to get it in a pure form.

Effect of heating. Heating to 100° C. for thirty minutes does not destroy the toxin, as the following protocols show.

Guinea-pig 58. Weight, 252 grams. Intraperitoneal inoculation of 30 mg. toxin No. 11 heated 30 minutes in solution, in a sealed tube in boiling water. Guinea-pig found dead in fifteen hours. Autopsy showed typical picture of toxin action.

Guinea-pig 63. Weight, 297 grams. Intraperitoneal inoculation of 30 mg. toxin No. 11 heated 30 minutes at 70° C. in a sealed tube. Found dead in fifteen hours. Autopsy showed typical picture of toxin action.

Guinea-pig 62. Control. Weight, 298 grams. Intraperitoneal inoculation of 30 mg. toxin No. 11. Dead in four and a-half hours.

Guinea-pig 66. Weight, 300 grams. Intraperitoneal inoculation of 28 mg. toxin No. 16 digested twenty-four hours with trypsin and heated 30 mintues in boiling water in a sealed tube. Found dead in twenty hours and autopsy showed typical picture of toxin action.

Guinea-pig 75. Control. Weight, 317 grams. Intraperitoneal inoculation of 28 mg. toxin No. 16 digested twenty-four hours with trypsin. Found dead in twenty hours and autopsy showed typical picture of toxin action.

DIGESTION OF PROTEUS TOXIN WITH TRYPSIN.

Digestion in a 0.25 per cent trypsin solution containing 0.15 per cent sodium carbonate does not destroy the toxic action as is shown in Table III. After digestion the solution was heated to the boiling point to destroy the enzyme.

TABLE III.

ANIMAL	WEIGHT	TOXIN NO.	AMOUNT INJECTED INTO PERITONEAL CAVITY	HOURS OF DIGESTION	RESULT
	grams		*milligrams*		
Mouse		13	2.5	No trypsin (Control)	Found dead in 23 hrs.
		13	2.5	3.75	Found dead in 20 hrs.
		13	2.5	9	Found dead in 14 hrs.
		13	2.5	24	Found dead in 23 hrs.
				Trypsin+ Na_2CO_3	Lived
		15	5	24	Dead in 48 hrs.
		16	5	24	Found dead in 15 hrs.
Guinea-Pig. No.					
69	360	15	24	0 (Control)	Dead in 42 hrs.
71	312	15	24	24	Dead in 6 hrs.
67	380	16	28	0 (Control)	Dead in 20 hrs.
75	317	16	28	24	Dead in 20 hrs.

DIGESTION WITH PEPSIN.

The toxin was made up with a 0.5 per cent pepsin solution in $\frac{N}{10}$ HCl and incubated for twenty-four hours before it was injected. Digestion was not complete, there being a residue left in the tube. In table IV the results of this experiment show that pepsin has as little effect as trypsin.

TABLE IV.

ANIMAL	TOXIN NO.	AMOUNT INJECTED INTO PERITONEAL CAVITY	HOURS OF DIGESTION	RESULT
		milligrams		
Mouse.........	15	5	Toxin + $\frac{N}{10}$ HCl (Control)	Found dead in 21 hrs.
	15	5	24	Dead in 3 days
	15	5	24	Dead in 2 days
Guinea Pig No. 73 Weight 310 g.	15	20	Toxin + $\frac{N}{10}$ HCl (Control)	Dead in 21 hrs.
70 Weight 305 g.	15	20	24	Dead in 21 hrs.

We evidently are dealing with a very stable substance as neither tryptic nor peptic digestion; $\frac{N}{10}$ HCl nor 0.15 per cent Na_2CO_3, acting for short periods of time, affect its toxic action. Dissolved in 1 per cent Na_2CO_3 the toxic properties were diminished but not lost.

An attempt was made to separate the toxin from the protein by adding enough HCl to make a $\frac{N}{10}$ solution, filtering off the precipitated protein and suspending it in salt solution. Injection of the precipitate and filtrate showed that the toxic substance had been carried down with the precipitate as is shown in Table V.

TABLE V.

ANIMAL	TOXIN NO.	AMOUNT OF TOXIN REPRESENTED IN INJECTION INTO PERITONEAL CAVITY	PORTION INJECTED	RESULT
		milligrams		
Mouse.........	15	5	Control	Dead in 20 hrs.
	15	5	Precipitate	Dead in 21 hrs.
	15	5	Precipitate	Dead in 3 days
	15	5	Filtrate	Alive
	15	5	Filtrate	Alive

EFFECT OF DIALYSIS.

In order, if possible, to separate the toxin from the protein, the solution was dialyzed in running water in a tube, the end of which was covered by parchment paper. The tube was shaken from time to time and before injection the fluid inside was brought back to its original level.

TABLE VI.

ANIMAL	TOXIN NO.	AMOUNT INJECTED INTO PERITONEAL CAVITY	HOURS OF DIALYSIS	RESULT
		milligrams		
Mouse	13	2.5	12	Dead in 5.5 hrs.
	13	2.5	72	Dead in 20 hrs.
	16	5.0	48	Dead in 15 hrs.
Guinea-pig No. 54 Weight 407 g.	16	28	60	Dead in 21 hrs.

Since the substance is non-dialyzable we must conclude that it is of a complicated chemical structure or that it is firmly bound to or is a part of the protein.

In order to separate, if possible, the toxin from the protein, we next tried the effect of dialysis after digestion with trypsin and pepsin, but the results were not as definite as in the preceding experiments. In the majority of cases after dialysis for two or three days the toxin was present inside the parchment paper in undiminished strength. In one case after digestion for twenty-four hours with trypsin and dialyzing seventy-two hours, 20 mgs. killed a guinea-pig, weighing 290 grams, in sixteen hours and autopsy showed a typical picture of toxin action.

EFFECT OF OXIDATION ON THE TOXIN.

The effect of oxidation on the toxic substance was tried by adding Parke, Davis and Company's hydrogen dioxide solution as follows:

Tube A. Control. 2 cc. of a 0.5 per cent solution of toxin 17 (digested twenty-four hours with trypsin) + 0.5 cc. salt solution.

Tube B. 2 cc. toxin as in A. + 0.5 cc. H_2O_2 solution.

Tube C. 2 cc. toxin as in A. + 0.5 cc. H_2O_2 solution + small crystal of $FeSO_4$.

The tubes were incubated at 37° C. for two hours and at the end of this time the oxidation in tube C was very marked. Mice were inoculated, each mouse receiving what corresponded to 5 mg. of the dried toxin into its peritoneal cavity.

TABLE VII.

INJECTION	MOUSE 1	MOUSE 2
Tube A. Toxin + salt solution (Control)......	Found dead in 21.5 hrs.	Found dead in 21.5 hrs.
Tube B. Toxin + H_2O_2	Found dead in 45 hrs.	Found dead in 45 hrs.
Tube C. Toxin + H_2O_2 + $FeSO_4$..............	Found dead in 50.5 hrs.	Lived.

This experiment shows that the toxin is partially destroyed by powerful oxidation.

IMMUNITY TOWARDS THE TOXIN.

Several attempts were made to establish an immunity in guinea-pigs toward the toxin by means of subcutaneous and sublethal intraperitoneal inoculations but these all failed. In some instances, where repeated, subcutaneous inoculations were made, the animal apparently became more sensitive towards the substance as the lesion appeared quicker and was more severe than in the first inoculation.

In rabbits the effect of sublethal intravenous inoculations was very interesting, as the following protocols show.

Rabbit No. 5.

August 5. Weight, 2160 grams. Intravenous injection of 10 mg. toxin No. 13. No apparent effect.

August 8. Weight, 1860 grams. Temperature, 38.5°. Intravenous injection of 10 mg. toxin No. 15.

August 9. Temperature, 39.2°. Rabbit not eating.

August 10. Temperature, 38.4°, weight, 1765 grams.

August 11. Temperature, 39.8°. Intravenous injection of 10 mg. toxin No. 15.

August 13. Weight, 1710 grams.

August 17. Weight, 1717 grams. Intravenous injection of 20 mg. toxin No. 16 digested twenty hours with trypsin.

Immediately after the inoculation the rabbit lay on its side and kicked rather violently. This kicking was not of a spasmodic character, however. Soon it became quiet and the breathing became gradually slower until it stopped altogether. The heart was beating a short time after the breathing stopped but the animal was dead in five minutes from the time it was inoculated.

Autopsy at once. Pupils slightly dilated.

Peritoneal Cavity. Liver swollen and slightly congested; surface very rough. Spleen slightly enlarged and bluish in color. Kidneys apparently normal. Adrenals apparently normal. The walls of the stomach appeared to be slightly thickened but this was not marked. Intestines normal.

Pleural Cavity. Heart empty and walls flabby. Left ventricle apparently normal. Lungs collapsed and very pale. No thrombi could be found in the pulmonary vessels.

Rabbit No. 4.

August 6. Weight, 1780 grams.

August 7. Intravenous injection of 10 mg. toxin No. 15. Immediately after the inoculation the rabbit appeared to be dizzy, falling on its side when it attempted to move. This effect passed off in a short time.

August 8. Rabbit appears to be quite sick and breathes with difficulty. Temperature, 38.3°, weight 1607 grams.

August 9. Temperature, 39.0°. Intravenous injection of 5 mg. toxin No. 15. After the inoculation the rabbit lay for about two hours on its side as though exhausted.

August 10. Temperature, 39.6°, weight 1525 grams.

August 11. Temperature, 40.4°. Intravenous injection of 5 mg. toxin No. 15.

August 13. Weight, 1515 grams.

August 17. Weight, 1575 grams.

August 20. Weight, 1607 grams.

August 22. Weight, 1560 grams. Rabbit had not been fed. Intravenous injection of 10 mg. toxin No. 17, digested 24 hours with trypsin. Immediately after the inoculation the rabbit fell from side to side and then lay and kicked rather violently, the kick becoming more feeble until it was quiet. The breathing was at first rapid and then gradually slowed until it had stopped altogether for quite an interval. Then the rabbit gave several deep gasps and died. During this time and for a short time after the last breath was taken the heart beat at first rapidly and then slower and slower until it stopped. The rabbit died five minutes after being inoculated.

Autopsy at once. Pupils normal.

Peritoneal Cavity. Liver pale and roughened. Spleen bluish in color and slightly swollen. Kidneys slightly, if any, congested. Adrenals normal. Digestive tract apparently normal.

Pleural Cavity. Lungs collapsed and apparently normal. No thrombi could be found. Heart showed white areas in the muscle wall of the right ventricle.

The fact that rabbits apparently became hypersensitive might indicate that an immunizing process is taking place, but we were never able to establish an immunity of such a degree that it would protect the animal against a fatal dose of the toxin.

In the guinea-pig the picture, in an animal dead from an intraperitoneal inoculation, is strikingly like that of the tuberculin reaction, and it may be that the two act in a similar manner.

On the other hand the effect produced in cats is very similar to that produced by Schmiedeberg's sepsin (25), as was pointed out by Levy (26). Faust (27) has repeated the work of the former and succeeded in isolating a crystalline substance to which he gives the formula,

$$CH_2NH_2.CHOH.CHOH.CH_2.CH_2NH_2.$$

His yield was very small being only 0.03 gram of the sepsin sulphate, for 5 kilos of putrefying yeast. Twenty milligrams of this substance caused in dogs, vomiting, bloody diarrhœa, and finally death. Our relatively impure substance, in a much smaller amount, caused similar effects in cats, and the autopsy showed the intense congestion of the digestive tract similar to that produced by sepsin. Faust succeeded in converting his sepsin into cadaverin by repeated boiling but we were unable to do this with the proteus toxin showing that the two substances are probably not the same.

Vaughan and his pupils (28) have made an extensive study of the so-called endotoxins of a number of organisms and in looking over their work one is struck with the similarity of the action of all these substances.

Through the kindness of Dr. Theobald Smith we were able to compare the colon toxin with that of proteus. A freshly isolated culture of B. coli was grown on large agar slants and the seven day growth precipitated with alcohol, the same as were our Proteus cultures. This substance, when dried, proved to be very toxic, 4.0 mg. per 100 gram of body weight killing a guinea-pig in less than twenty hours. It resisted heating to 100° for thirty minutes, tryptic and peptic digestion, the same as the proteus toxin. The picture in guinea-pigs differed in that there was less exudate into the peritoneal cavity and the venous congestion was not as marked with the colon as with the proteus toxin. Injected into the subcutis the necrosis was very slight. When injected intravenously into cats, the only effects we were able to observe were drowsiness, loss of appetite, and marked loss in weight. There was no vomiting nor diarrhœa. In rabbits an intravenous injection of 15 mg. of this colon toxin caused a drowsiness and a temporary loss in weight.

These precipitated cultures are probably made up of a great variety of substances, but it is conceivable that the cause of their toxic effects is a definite chemical group whose action is modified by a slight change in grouping or by the other substances present.

It should be noted that while this dried precipitate is in itself very toxic it comes from a relatively large amount of culture. One milligram from a bouillon culture represents 1 cc. of the culture and therefore a fatal dose for a 300 gram guinea-pig, 27 mg. represents 27 cc. of the bouillon culture. Myerhof (29) too noted the relative toxicity of living cultures of proteus to the dead bacteria, finding the living culture four times as toxic as the bodies of the bacteria and twenty times as toxic as the filtered cultures.

CONCLUSIONS.

1. Culturally, *Proteus vulgaris* varies greatly, its most constant property being the fermentation of dextrose and saccharose and its failure to ferment lactose.
2. In the absence of carbohydrates, proteus destroys some native albumins; and produces ammonia, primary amines, hydrogen sulphide, fatty acids of a high molecular weight, aromatic oxyacids, indol and indol-acetic acid. It does not produce phenol, skatol, mercaptan, alcohols, aldehydes, nor ketones.
3. *Proteus vulgaris* possesses both the properties of fermentative and putrefactive organisms.

4. Feeding experiments vary greatly and probably depend upon the virulence of the organisms fed and upon the diet.

5. The bodies of the bacteria precipitated by alcohol, contain a toxic substance which has the following properties:

a. It is thermo-stable.

b. It resists tryptic and peptic digestion.

c. It is non-dialyzable and is either firmly bound to or is a part of the protein of the cells.

d. It is partially destroyed by powerful oxidation.

e. Injected into the peritoneal cavity of guinea-pigs it causes rapid death, but when the same amount is injected into the subcutis an extensive necrosis is the result.

f. Intravenous injection in cats causes severe vomiting, bloody diarrhœa and death. In this respect it resembles sepsin but in other ways it differs so that we cannot conclude that they are the same.

g. Rabbits may become hypersensitive to repeated inoculations.

h. We were not able to establish an immunity in guinea-pigs or rabbits.

We wish to express our indebtedness to Drs. Theobald Smith, H. D. Dakin and Alfred J. Wakeman for their advice and aid that they so freely gave throughout this work.

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